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13. ABSTRACT (Maximum 200 Words)

In prostate cancer, constitutive activation of NFκB and AP1, non-functional p53 and defective androgen receptor signaling together may play a role in rendering intrinsic resistance to radiation in prostate cancer. Many reports in literature have showed that Curcumin (diferulylmethane is a major chemical component of a curry spice, turmeric) is a potent inhibitor of prostate cancer cell growth. It was found that Curcumin inhibits TNF-α mediated activation of NFκB and down-regulates Bcl-2 expression. From this reported observation, we hypothesized that Curcumin will abrogated the upregulation of pro-survival genes by radiation. Our preliminary data demonstrated that Curcumin significantly inhibited the growth of androgen-independent prostate cancer cell line PC-3. In addition, Curcumin conferred significant enhancement of radiation-induced clonogenic inhibition and apoptosis in prostate cancer cell line PC-3. Interestingly, Curcumin also inhibited the radiation induced prosurvival factors such as NFκB activity and Bcl-2 expression. These results underscore the need to formally study the functional relevance of Curcumin in synergizing the effects of radiation in prostate cancer.
Since the proposal was funded for two years and the PI was asked to drop aim 3 and request to resubmit the aims. Hence, the first aim is completed in the proposed time.

14. SUBJECT TERMS
Curcumin, radiation, prostate cancer, apoptosis

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Table of Contents

Introduction

Inhibition of radiation-induced pro-survival genes by Curcumin in prostate cancer.

PI: Damodaran Chendil., PhD

BACKGROUND: In prostate cancer, constitutive activation of NFκB and AP1, non-functional p53, defective androgen receptor signaling, and high constitutive levels of Cycloxygenase (COX-2) enzyme and inactivation of tumor suppressor gene PTEN/MMAC together may play a role in rendering intrinsic resistance to radiation in prostate cancer. In wild-type p53 background, radiation often induces p53 that culminates the upregulation of Bax with a concomitant decrease in the level of Bcl-2 leading to cell death. On the other hand, in p53 mutant background, radiation causes induction of bcl-2 through activation of NFκB. Many reports in literature have showed that Curcumin (diferulylmethane is a major chemical component of a curry spice, turmeric) is a potent inhibitor of prostate cancer cell growth. It was found that Curcumin inhibits TNF-α mediated activation of NFκB and down-regulates Bcl-2 expression. From this reported observation, we hypothesized that Curcumin will abrogated the upregulation of pro-survival genes by radiation. Our preliminary data demonstrated that Curcumin significantly inhibited the growth of androgen-independent prostate cancer cell line PC-3. In addition, Curcumin conferred significant enhancement of radiation-induced clonogenic inhibition and apoptosis in prostate cancer cell line PC-3. Interestingly, Curcumin also inhibited the radiation induced pro-survival factors such as NFκB activity and Bcl-2 expression. These results underscore the need to formally study the functional relevance of Curcumin in synergizing the effects of radiation in prostate cancer.

HYPOTHESIS: Based on these factorial observations mediated above, it is hypothesized that in prostate cancer cells that lack functional p53, radiation will upregulate NFκB activity and Bcl-2 expression and confer an "induced radiation resistant phenotype". Curcumin will inhibit the radiation-induced NFκB and Bcl-2 expression and will allow radiation to enhance the apoptosis. Thus, we hypothesize that addition of Curcumin will abrogate the "induced radiation resistance" phenotype and enhanced pro-apoptotic action of radiation in prostate cancer. **SPECIFIC AIMS:** To test the above hypotheses, two specific aims are proposed:

(1) Effect of Curcumin with or without radiation on the clonogenic inhibition, cell cycle phase and apoptosis of prostate cancer cell lines.

(2) To determine the mechanism of action of Curcumin mediated radiosensitizing effect by analyzing the pro-survival transcription factor activity (such as AP-1, NFκB and AR activation) and its target genes.

STUDY DESIGN: Specific aims 1 and 2 will be studied using PC-3, DU-145, 22Rv -1 and LNCaP cells. In aim 1, we will study the effect of Curcumin with or without radiation on clonogenic inhibition and cell cycle phases and correlate with apoptosis. In the second aim, we will study the kinetics of pro-survival transcription factor activity such as AP1, AR and NFκB in response to Curcumin in combination with and without radiation. This will be analyzed by protein-DNA array and confirmed gel shift analysis and reporter assays.

RELEVANCE: Curcumin is a natural dietary compound that is not consumed in USA. On the contrary, everyday use of Curcumin as a dietary agent in India may have led to reduce incidence of prostate cancer. In addition to chemopreventive effects of Curcumin on prostate cancer, it kills prostate cancer cells and is a potent chemo-adjuvant for enhancing the effects of radiation. Thus, if our *in-vitro* studies demonstrate that Curcumin plays pivotal role in enhancing the effects of radiation, then we will plan studies to test this concept in animal models and patients with prostate cancer.

Final report.

BODY

In this section a detailed description of the research accomplishment will be described as per outlined in the approved states of work. The following tasks pertain to the fist year of the grant period.

TASK-1. Effect of curcumin in PC-3, DU-145, 22Rv1 and LNCaP cells, months 1-12

Dose response curves for curcumin and curcumin plus radiation will be generated for all the four prostate cancer cell lines. In response to these treatments, cell death will assayed by TUNEL staining and flow cytometry (Propidium Iodide staining). Cell cycle changes will be assessed by flow cytometry.
The response data on clonogenic inhibition and apoptosis will be compared with cell cycle changes. Months 1-12.

Aim 1: Effect of Curcumin with or without radiation on the clonogenic inhibition, cell cycle assays and apoptosis of androgen dependent and androgen independent prostate cancer cell lines.

Purpose: The present study was taken to determine that curcumin is a potent sensitizer of radiation-induced pro-apoptotic action in prostate cancer cells. This was accomplished by partially by aim 1.

Results: Curcumin (diferuloylmethane) is a major chemical component of turmeric (*Curcuma longa*) and is used as a spice to give a specific flavor and yellow color in Asian food. Curcumin exhibits growth inhibitory effects in a broad range of tumors as well TPA-induced skin tumors in mice. This study was undertaken to investigate the radio sensitizing effects of curcumin in prostate cancer cell lines. Results of PC-3 results are given in abstract form and these results are published in peer reviewed journal (Oncogene) and a copy of published article is enclosed herewith in Appendix II along with the figures.

PC-3

Curcumin [Diferuloylmethane] is a major chemical component of turmeric [*Curcuma longa*] and is used as a spice to give a specific flavor and yellow color in Asian food. Curcumin exhibits growth inhibitory effects in a broad range of tumors as well as in TPA-induced skin tumors in mice. This study was undertaken to investigate the radiosensitizing effects of curcumin in p53 mutant prostate cancer cell line PC-3. Compared to cells that were treated with curcumin alone (fig-1 and table-1) or irradiated alone (SF_2=0.635; D_0=231cGy) (Fig-2), curcumin at 2 and 4 μM concentrations in combination with radiation showed significant enhancement of radiation induced clonogenic inhibition (SF_2=0.224: D_0=97cGy and SF_2=0.080: D_0=38cGy) (Table 1) and apoptosis (fig 3A & 3B). Curcumin caused a strong G_2/M block which is an important phase sensitive to radiation. Curcumin treated cells showed 32.55% of G_2/M block at 12 hours, 48.10 % at 24 hours, 42.75% at 48 hours and 35.89% at 72 hours. (Table-2) It has been reported that curcumin inhibits TNF-α induced NFκB activity that is essential for Bcl-2 protein induction. In PC-3 cells, radiation up-regulated TNF-α protein leading to an increase in NFκB activity resulting in the induction of Bcl-2 protein. However, curcumin in combination with radiation treatment had shown inhibition of TNF-α mediated NFκB activity resulting in bcl-2 protein down regulation. Bax protein levels remained constant in these cells after radiation or curcumin plus radiation treatments. However, the down regulation of Bcl-2 and no changes in Bax protein levels in curcumin plus radiation treated PC-3 cells, together, altered the Bcl2:Bax ratio and this caused the enhanced radiosensitization effect. In addition, significant activation of cytochrome-c, caspase-9 and

caspase-3 were observed in curcumin plus radiation treatments. Together, these mechanisms strongly suggest that the natural compound curcumin is a potent radiosesitizer, and it acts by overcoming the effects of radiation induced pro-survival gene expression in prostate cancer.

DU-145

Different concentrations of curcumin (2 μM, 4μM and 5 μM) enhance the radio sensitization effect on DU-145 cells (Table 1) compared to curcumin alone (fig-4) or radiation alone (fig-5). In combination with radiation, curcumin induced a significant induction of apoptosis (31% at 24 h and 69% at 48 h) compared to radiation alone 4.1% at 24 h and 10.8% at 48 h) or curcumin alone (12% at 24 h and 23.08% at 48 h) (fig-6).There were no significant changes observed in cell cycle assays either treatment with curcumin alone or radiation alone or incombination.(Table-3)

22Rv1

As shown in table one, 22Rv1 cells are very sensitive to curcumin (Do=1.97 μM)(fig-7) and radiation (SF_2 =0.29 and D_o= 1.86 Gy) (fig-8). In clonogenic inhibition assays radiation and curcumin in combination, these cells even in lower concentrations showed more sensitivity compare to other four cell lines (PC-3, DU-145, LN3 and LNCaP). In addition curcumin enhances radiation induced apoptosis in 22Rv1 cells (fig-9 & fig-10). Interestingly, curcumin abrogates radiation induced G_2/M block in 22Rv-1 cells (table-4).

LNCaP

As we mentioned earlier, LNCaP cells were failed to form colonies, hence we used MTT assay to detect the cytotoxic effect of curcumin in this cell line. Curcumin (Fig-11) or radiation alone (fig-12) induced cytotoxicity in LNCaP cells, however incombination no significant cytotoxicity effects were observed (Fig-12). Similarly apoptotic assays demonstrated that curcumin or radiation alone induced significant amount of apoptosis compared incombination with curcumin and radiation. (Fig-13 &fig 14) There were no changes in cell cycle pattern in LNCaP cells treated either with radiation or curcumin or incombination. (Table-5)

Second year:

TASK-2. The functional role of pro survival transcription factors on the mechanism of action of Curcumin with and without radiation on prostate cancer cell lines, Months 1-24:

An array of 54 -transcription factor activity will be screened using Protein-DNA array kit purchased from Panomics Inc. Results obtained from array will be confirmed by gel shift and reporter assays.

In particular, AP1, NFκB and AR activity will be tested using gel-shift and reporter assays in cells exposed to Curcumin with or without radiation.

Aim-2 the functional role of pro survival transcription factors on the mechanism of action of Curcumin with or without radiation on prostate cancer cell lines.

Rationale: Based on the reasoning aforementioned in the background, it is hypothesized that in prostate cancer cells that lacks functional p53, radiation may up regulate pro-survival factors such as NFκB activity and Bcl-2 expression and confer an "induced radiation resistant phenotype Curcumin inhibit radiation-induced pro-survival factors such as AP-1, NFκB and Bcl-2 expression that will lead to enhanced radiation-induced apoptosis.

In this aim we used Protein-DNA array kit to find out whether curcumin regulates any transcriptional factors resulting curcumin mediated radiosensitization effect on prostate cancer cells. For this study we used PC-3 and LNCaP cell lines, and results revealed no additional transcriptional factors in these cell lines. Hence, support from University of Kentucky, we used RNA based microarray studies, to find out new genes which is regulated by curcumin. We used four groups (untreated, curcumin alone, radiation alone and in combination with curcumin and radiation) (Fig-15 and table-6) and these results confirmed that NFkB and AP1 are significantly down regulated in AR independent prostate cancer cell lines (PC-3 and DU-145) (Fig 16 and 17) than AR dependent prostate cancer cell lines (LNCap and 22RV1) (Fig 18 and 19). AR dependent prosatate cancer cell lines demonstrate very low or no detectable basal levels of NFkB and AP-1 were seen.

PC-3 and DU-145:
In both cell lines the endogenous NFkB and AP-1 levels are high and curcumin down regulated the basal levels in both cell lines and radiation upregulated both the pro-survival factors (NFkB and AP-1) and combination with radiation and curcumin down regulated both NFkB and AP-1 activation in both the cell lines (figure-16 and 17). Further, DHT induced AR activation was abolished in combination with curcumin.

LNCap and CWR22RV-1: As stated about in both cell lines no significant induction of radiation induced NFkB and AP-1 activation were not seen. (figures-18 and 19) Also there is no significant activation AR activation by DHT were seen.

Key Research Accomplishments:

1. Curcumin sensitizes prostate cancer cells and induced apoptosis in all the proposed prostate cancer cell lines. In 22Rv1 and PC-3 cells curcumin inhibits G_2/M block that is an important phase of cell cycle. Curcumin enhanced radiosensitized prostate cancer cell lines (except LNCaP).
2. Curcumin down regulated NFkB, AP-1 and AR activation resulting radiation induced resistance were completely abolished.
3. No significant changes of other pro-survival factors were not seen curcumin treatment in prostate cancer cell lines by DNA-protein arrays.
4. Curcumin specifically target prostate cancer cells without causing any toxicity to normal prostate epithelial cells.

Reportable outcomes

1. Presentations were made in the National meetings:

1. Chendil.D, Rama.S, David.M, Sathishkumar.S, Inayat.S.M, Dey.S, Mohiuddin.M, and Ahmed.M.M. **Inhibition of radiation-induced pro-survival genes by Curcumin in prostate cancer.** Presented at 94th annual conference of American Society for Cancer Research (2003), Washington.

2. R.S Ranga and D.Chendil. **Molecular effects of curcumin on prostate cancer.** Presented at University of Kentucky International student symposium (2003)

2. Publications.

Damodaran Chendil, Rama S Ranga, David Meigooni, Sabapathi Sathishkumar and Mansoor M Ahmed. Curcumin confers radio-sensitizing effect in prostate cancer cell line PC-3. **Oncogene (2004 23, 1599-1707.**

Conclusions

Our results suggest that curcumin specifically targets prostate cancer cells, without causing any toxicity to normal prostate epithelial cells. In addition, curcumin act as potent radiosensitizer by abrogating radiation induced G2/M block of cell cycle and other hand it down regulated NFkB, AP1 and AR activation in prostate cancer cell lines.

Appendix-1

Figure-1 Curcumin induces clonogenic inhibition in PC-3 cells:

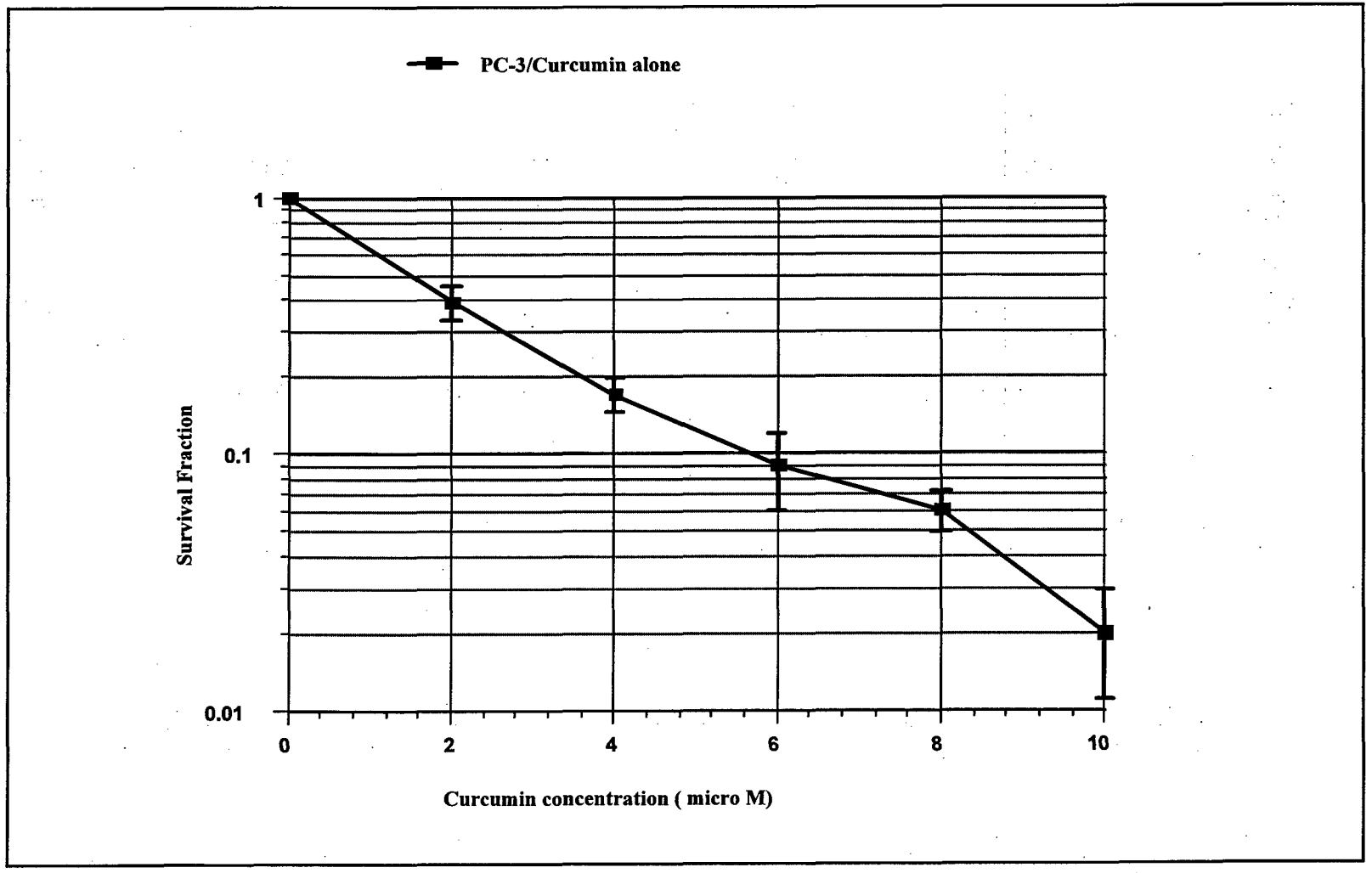


Figure-2 Curcumin enhances radiation induced clonogenic inhibition in PC-3 cells

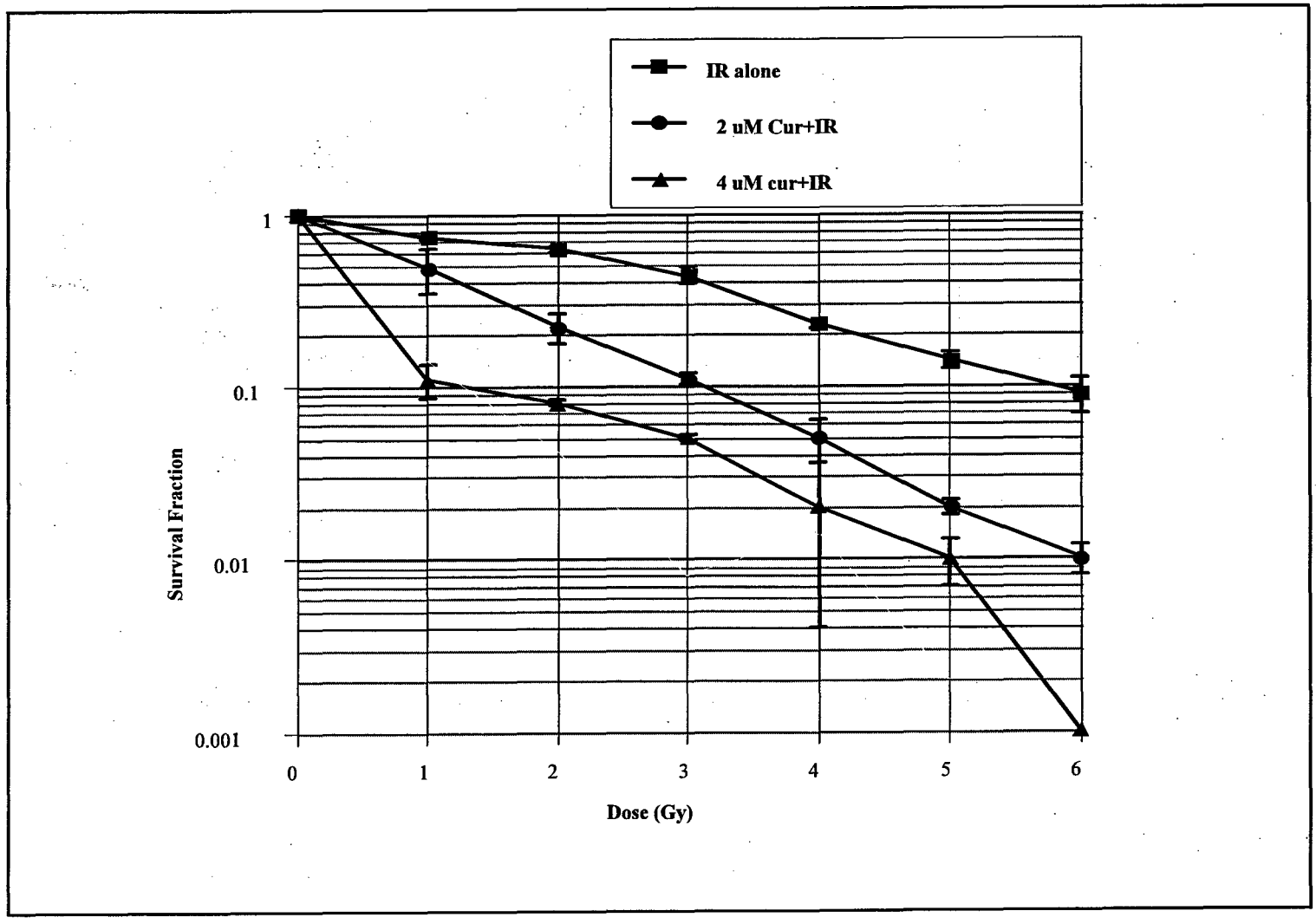


Table-1 SF_2,D_O values of curcumin, radiation and incombination treatments on prostate cancer cells.

Cell line	Treatment	SF_2 (Gy)	SF_2 ER	D_0	D_0 ER	ER
PC-3	IR alone	0.635	-	2.31Gy	-	-
	IR+2μMCur	0.224	2.83	0.97Gy	2.38	5.21
	IR+4μMCur	0.080	7.93	0.38Gy	6.07	14.0
DU145	IR alone	0.51	-	2.74Gy	-	-
	IR(2Gy)+2μMCur	0.33	1.545	-	-	1.545
	IR(2Gy)+4μMCur	0.20	2.55	-	-	2.55
	IR(2Gy)+5μMCur	0.09	5.66	-	-	5.66
	Curcumin alone	-	-	2.97μM	-	-
	Cur +IR(2Gy)	-	-	2.52μM	1.18	1.18
LN3	IR alone	0.40	-	2.07Gy	-	-
	Curcumin alone	-	-	1.61μM	-	-
	IR +0.25μMCur	0.27	1.48	1.08Gy	1.90	3.38
22rv1	IR alone	0.29	-	1.86Gy	-	-
	Curcumin alone	-	-	1.97μM	-	-

Figure 3 Curcumin enhance radiation induced apoptosis in PC-3 cells:

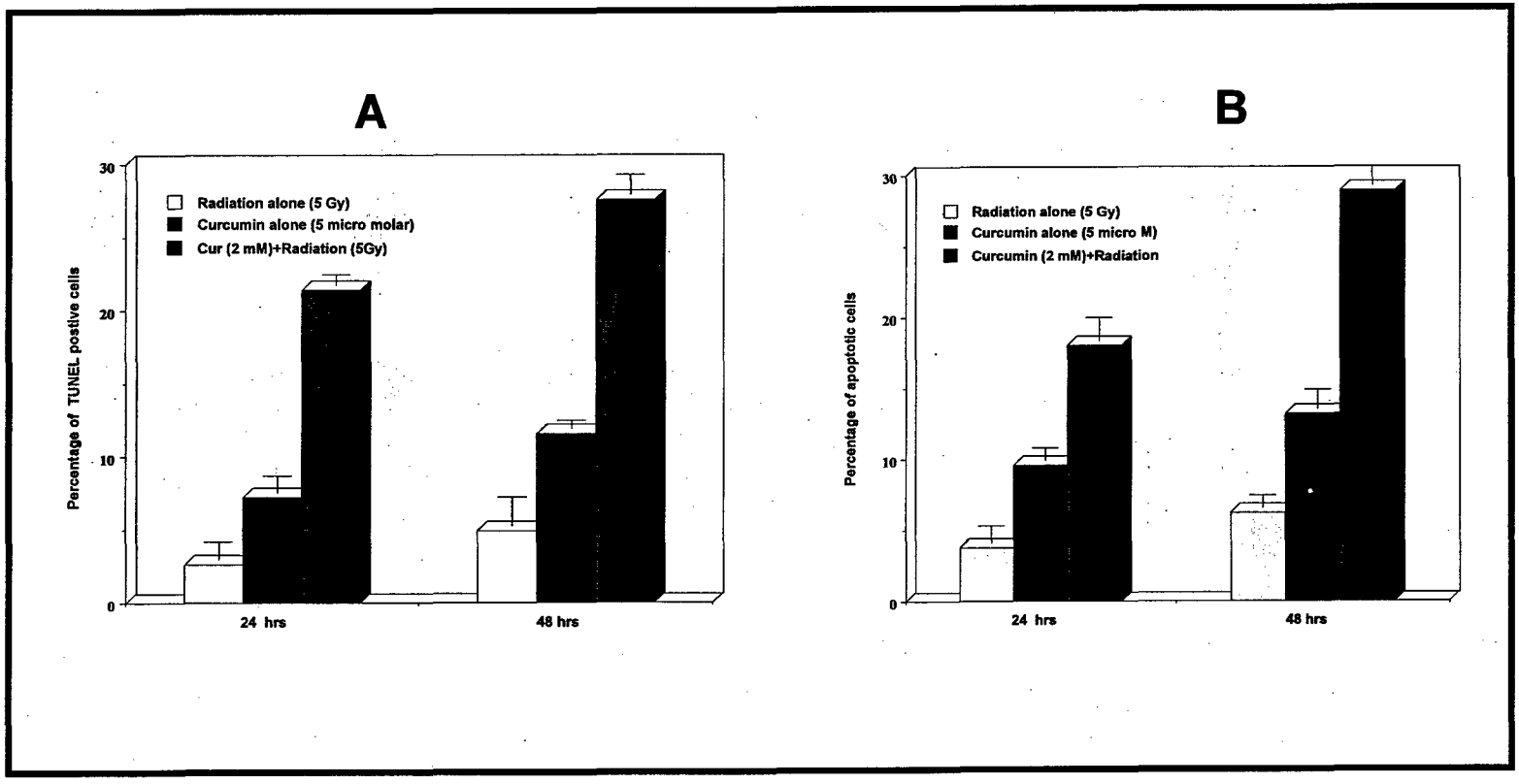


Table-2 Effects of curcumin or Ionizing radiation or in combination on cell cycle distribution in PC-3 cells

Cell cycle	Treatments	12 hours	24 hours	48 hours
G0/G1	Untreated	55.45 ±2.21	56.53 ± 4.21	53.23 ± 4.28
	curcumin alone	28.45 ±3.43	18.65 ± 3.76	20.05 ± 1.92
	radiation alone	15.62 ±2.34	21.61 ± 1.72	36.11± 2.69
	IR+Cur	24.21 ±3.99	25.22 ± 2.24	20.85 ± 1.65
S	Untreated	24.21 ±3.05	21.32 ±5.11	28.39 ± 3.76
	curcumin alone	39.00 ±1.98	33.25 ±1.07	37.20 ± 4.56
	radiation alone	35.47 ± 2.98	13.25 ± 1.09	22.86 ± 3.30
	IR+Cur	48.32 ± 3.12	44.20 ± 5.12	54.65 ± 5.74
G2-M	Untreated	20.34 ± 3.67	22.15 ± 1.98	18.38 ± 2.01
	curcumin alone	32.55 ±3.10	48.10± 2.16	42.75 ± 3.21
	radiation alone	48.82 ± 2.73	65.14 ± 5.92	41.03 ± 4.19
	IR+Cur	27.48 ± 2.12	30.58 ± 4.21	24.50 ± 1.99

Figure-4 Curcumin induces clonogenic inhibition in DU-145 cells

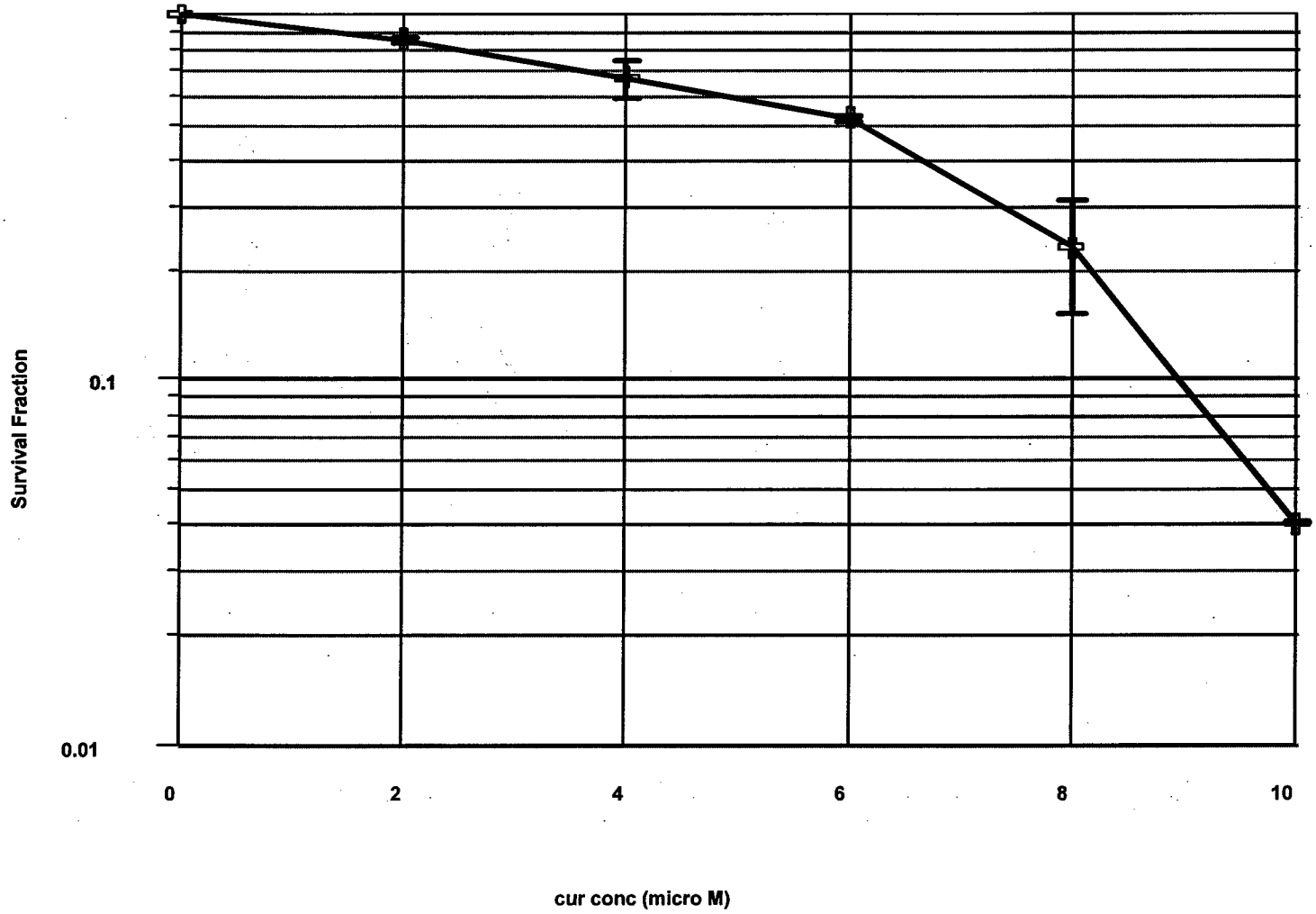


Figure- 5 Curcumin enhances radiation induced clonogenic inhibition in DU-145 cells

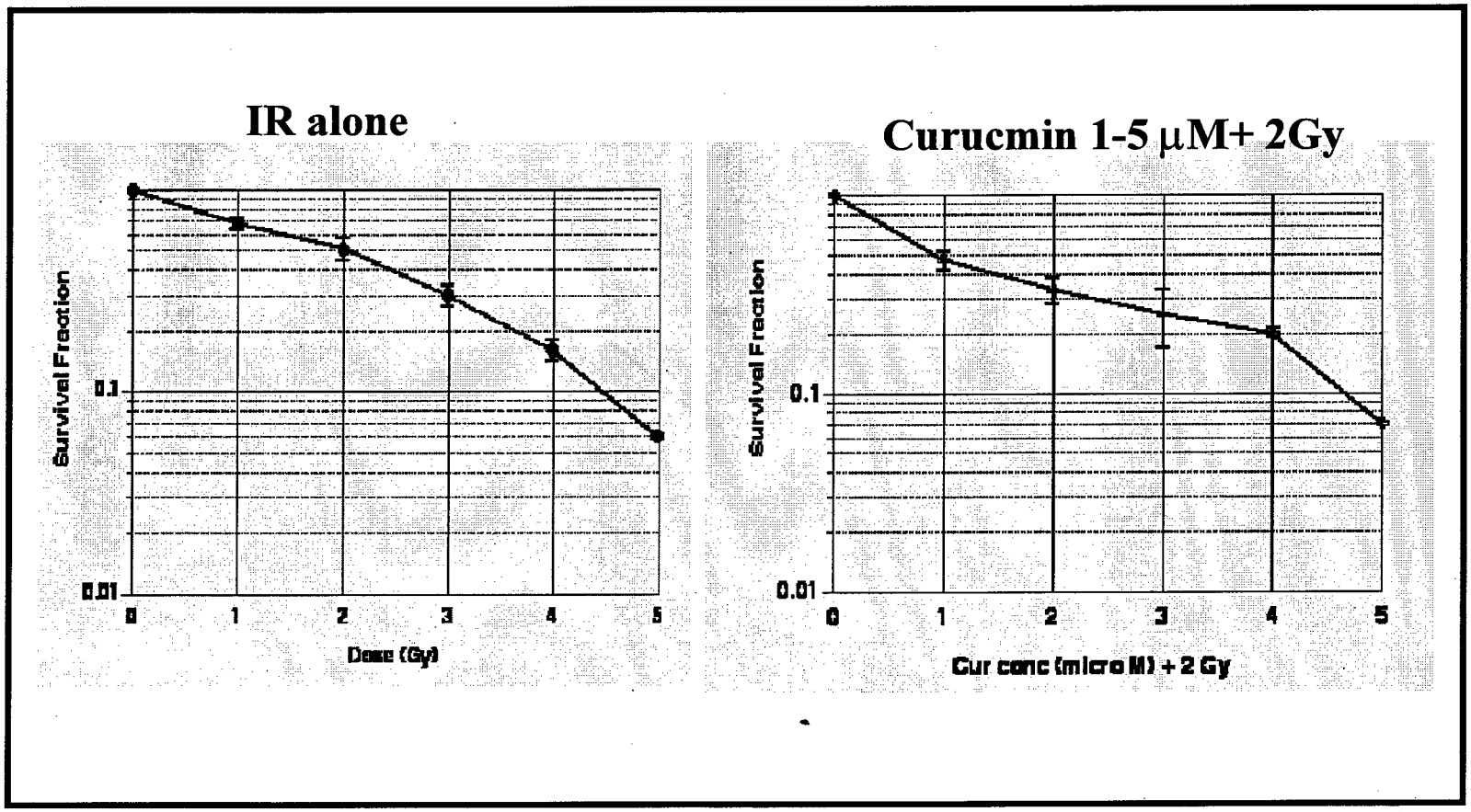
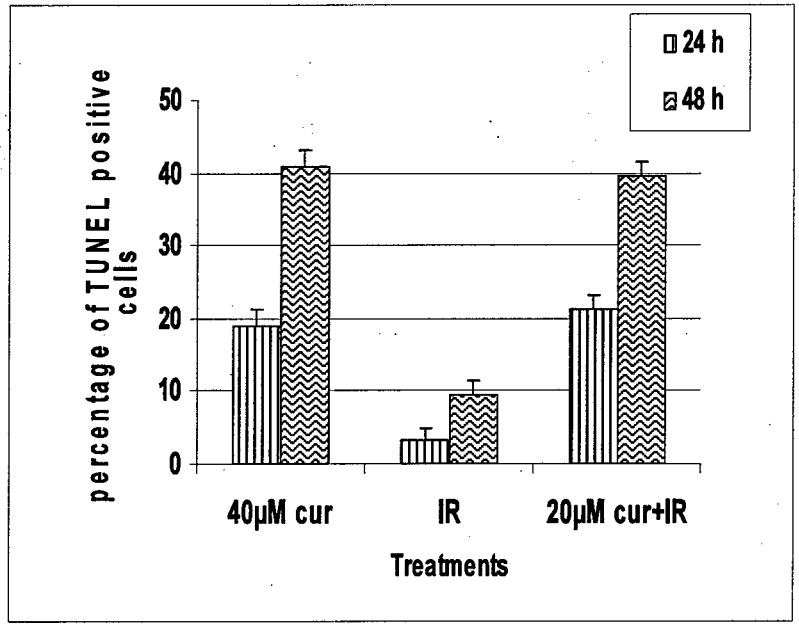
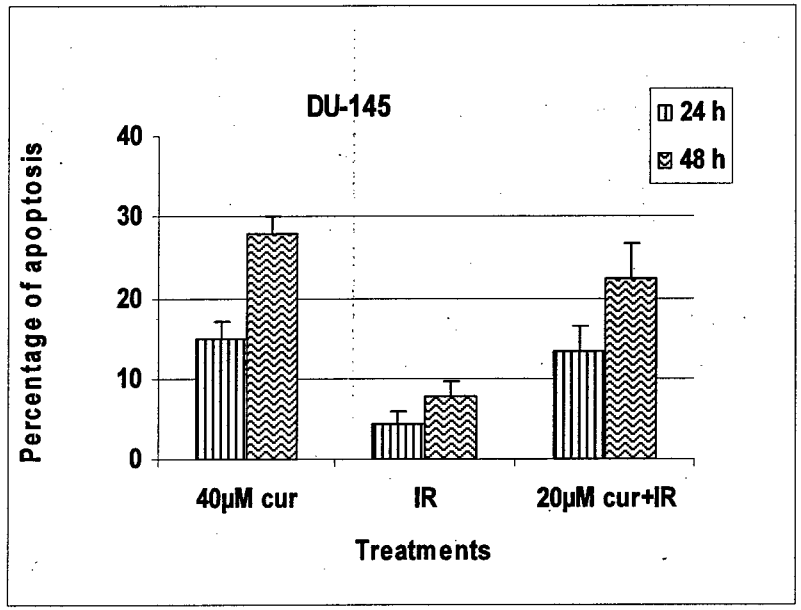


Figure 6 Curcumin enhance radiation induced apoptosis in DU-145 cells



A. TUNEL method



B. Annexin V-FITC method

Table-3 Effects of curcumin or Ionizing radiation or in combination on cell cycle distribution in DU-145 cells

	Treatment	6hrs	12hrs	24hrs	48 hrs	72hrs
G0/G1	UT	31.2±2.0	34.1±1.9	36.1±1.4	57.7±3.0	53.3±2.9
	Cur 10 µM	37.8±3.2	35.1±1.7	24.2±2.2	28.7±3.7	43.3±5.0
	15Gy	26.4±2.7	15.5±2.6	30.2±3.7	32.7±1.8	39.4±2.6
	Cur 10 µM+ 5 Gy	31.9±2.8	19.8±1.0	12.1±4.6	20.6±1.1	31.0±4.8
S	UT	22.9±3.7	26.0±4.2	27.3±4.2	11.1±5.0	9.7±.7
	Cur 10 µM	24.3±1.6	25.2±2.4	28.1±2.0	46.3±2.9	9.9±1.0
	5Gy	31.1±1.2	31.7±3.7	16.6±3.5	33.6±4.2	12.6±.8
	Cur 10 µM+ 5Gy	25.97±2.9	30.9±4.0	32.5±1.0	32.7±1.5	03.1±.0
G2/M	UT	45.9±2.7	40.1±1.8	36.5±2.3	31.2±2.4	36.9±1.6
	Cur 10 µM	37.9±1.4	39.7±1.5	47.6±3.1	25.0±1.3	46.7±5.4
	5Gy	42.6±2.1	53.1±3.2	53.2±1.4	33.6±3.8	47.9±2.6
	Cur 10 µM+ 5Gy	42.1±1.6	49.3±2.7	55.3±2.7	46.6±3.6	65.8±4.6

Figure-7 Curcumin induces clonogenic inhibition in 22Rv-1 cells

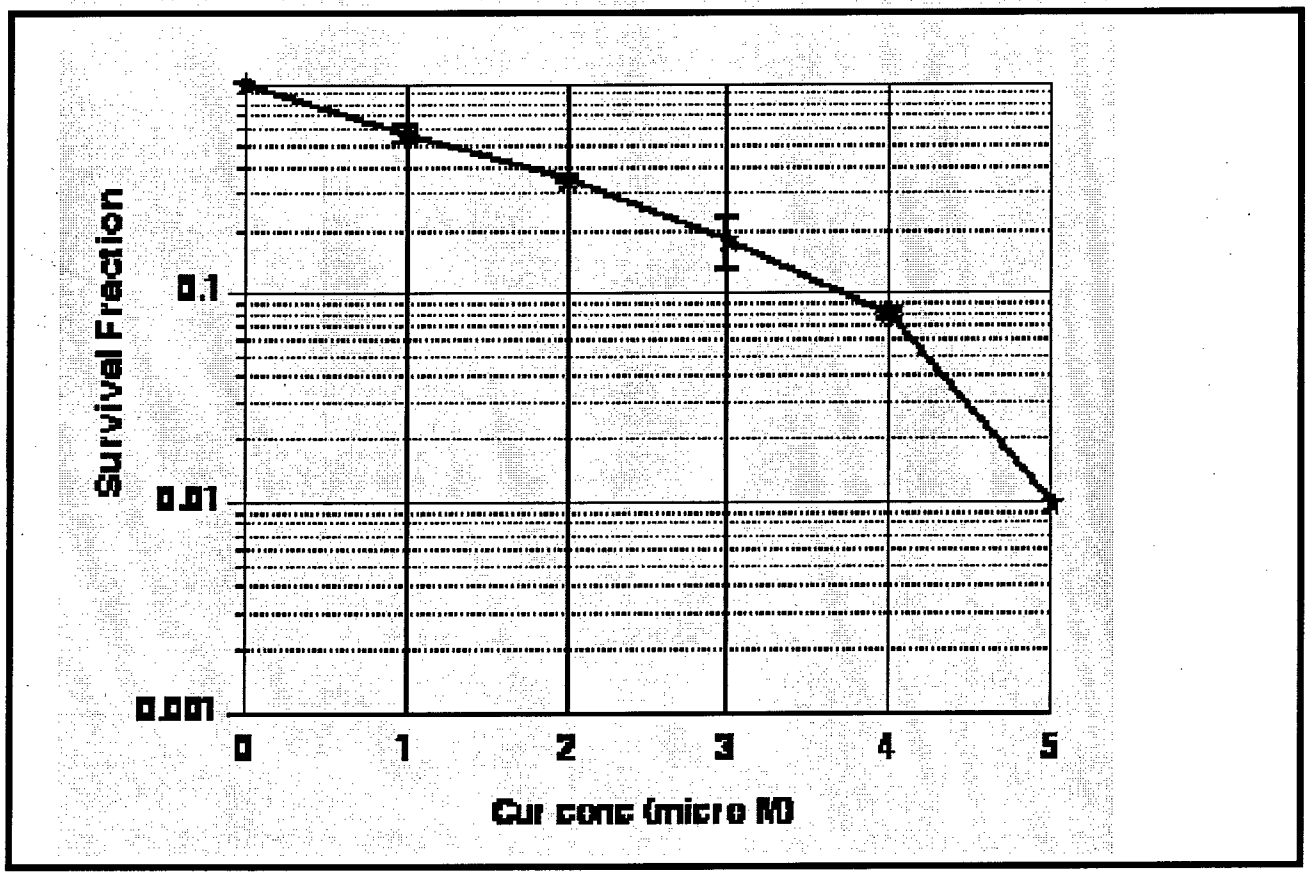


Figure-8 radiation induced clonogenic inhibition in 22Rv-1 cells

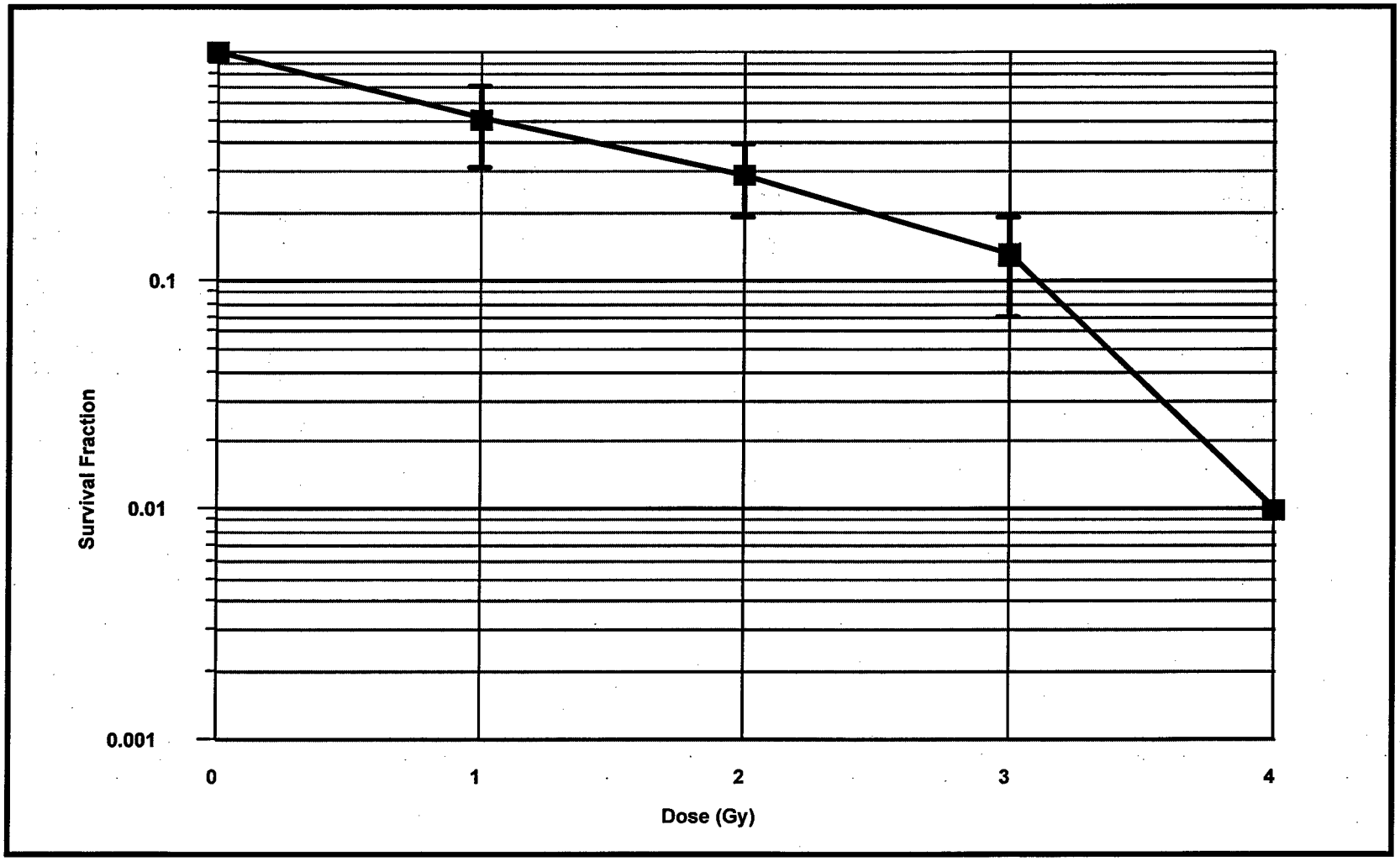


Figure 9 Curcumin enhances radiation induced apoptosis in 22Rv-1 cells By TUNEL

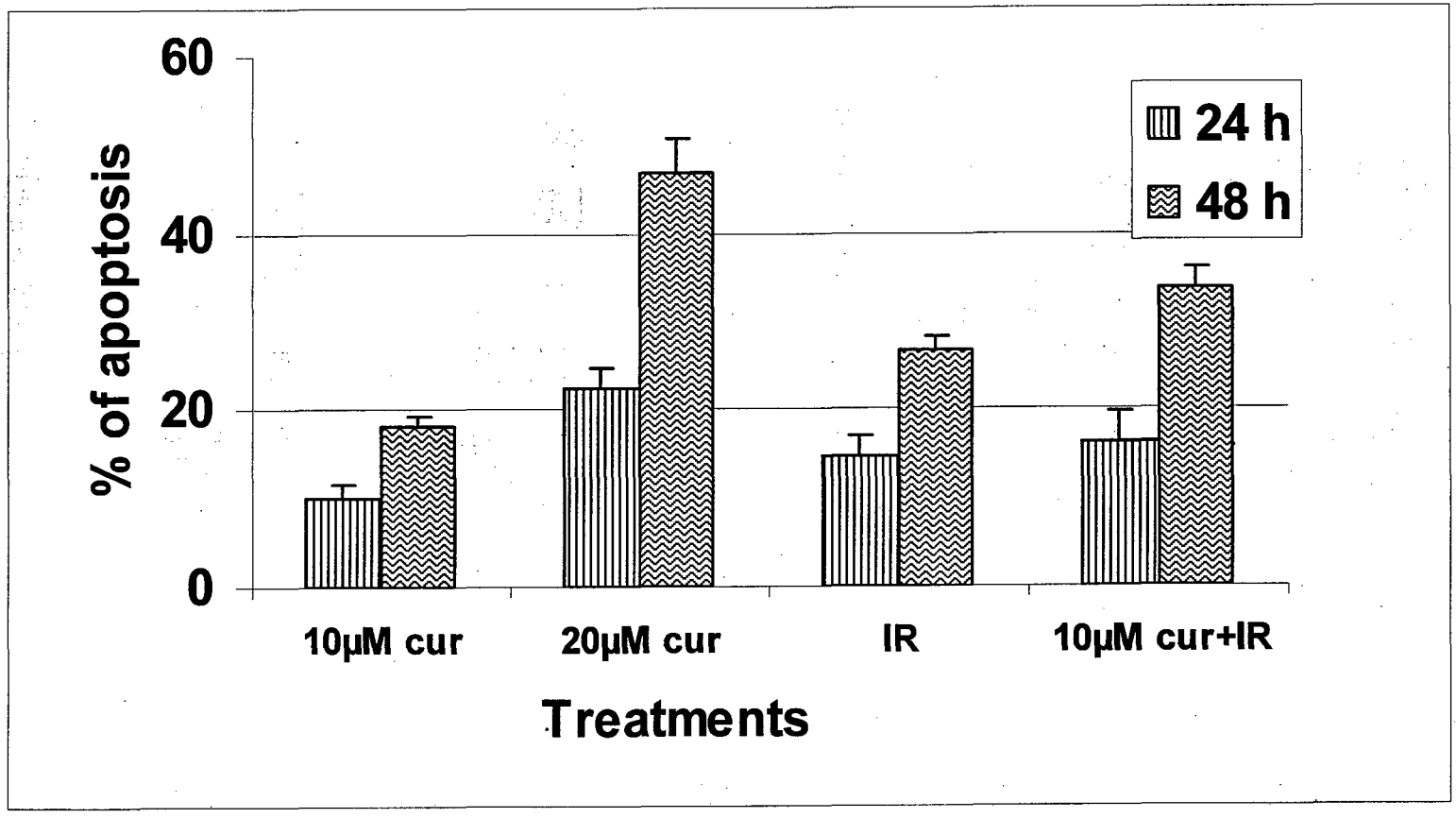


Figure 10 Curcumin enhances radiation induced apoptosis in 22Rv-1 cells By Annexin V-FITC

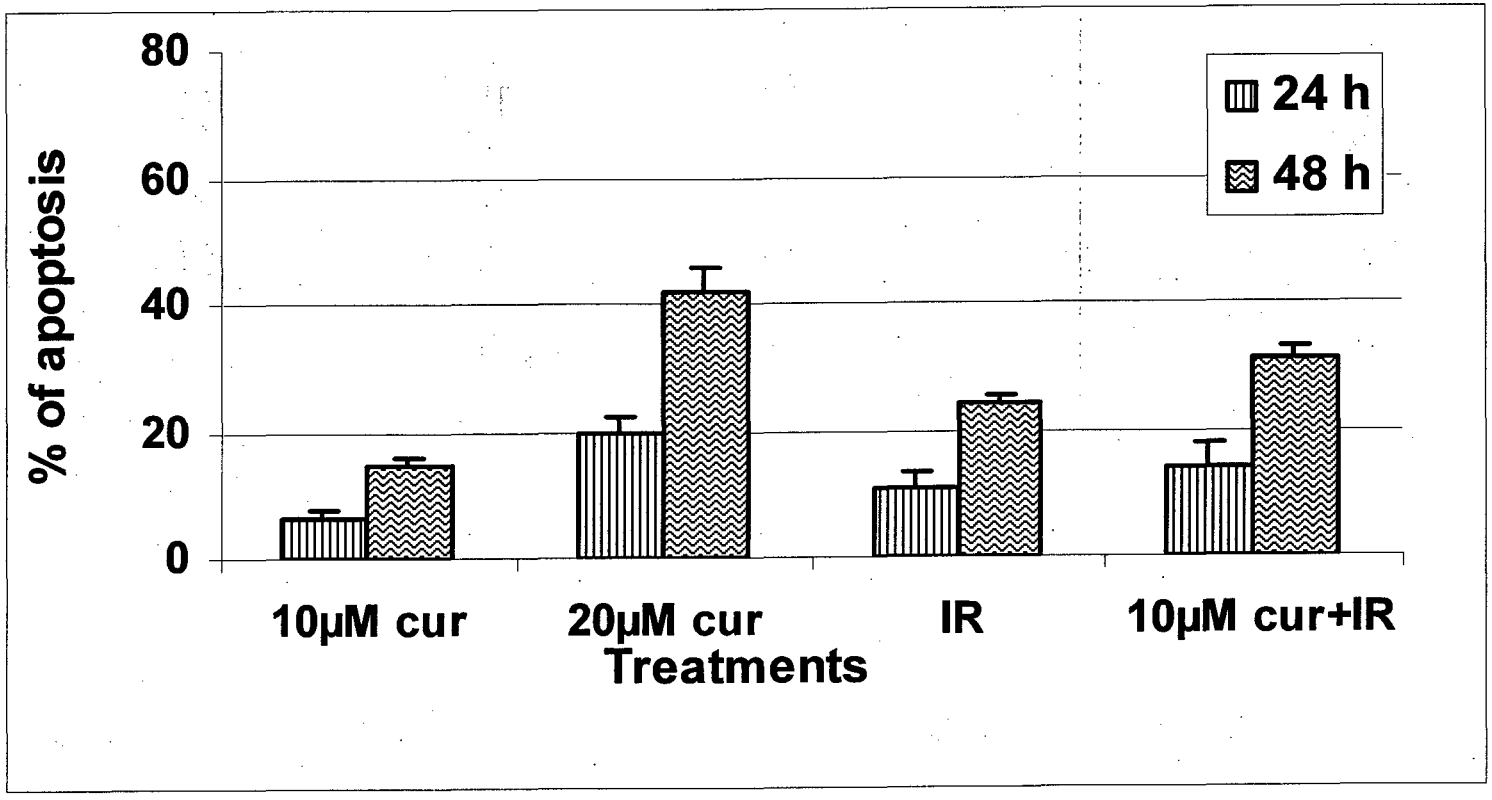


Table-4 Effects of curcumin or Ionizing radiation or in combination on cell cycle distribution in 22Rv-1 cells

	Treatment/	6hrs	12hrs	24hrs	48hrs	72hrs
G0/G1	UT	46.0 ±2.9	43.3±4.2	38.5±3.7	36.6±1.8	59.7±1.0
	Cur 20 µM	45.3 ±3.7	57.1±3.1	37.4±2.1	51.1±2.7	39.5±1.5
	5Gy	35.1 ± 4.0	24.1± 2.8	38.9±0.8	37.2±2.1	67.7±3.0
	Cur 20 µM+ 5Gy	43.2 ±2.1	31.8±1.6	29.6± 2.5	45±1.8	51.2±2.6
S	UT	44.9± 4.0	46.8± 3	49.9± 2.6	49.1±4.5	28.9±2.8
	Cur 20 µM	38.7±3.7	30.3±2.3	48.4±1.2	38 ±3.2	45.5±3.6
	5Gy	40.8± 3.5	37.3±4.6	26.1±4.2	32.8±1.6	11.5±2.6
	Cur 20 µM+ 5Gy	36.1±1.9	48.2±5.1	39.5±5.4	33.8±1.0	30.9±1.5
G2/M	UT	09.1± 2.6	10.1±1.9	11.6±1.9	14.3±2.4	11.2±1.8
	Cur 20 µM	16.2± 2.9	11.7±2.1	14.2±3.2	10.0±3.1	14.8±1.9
	5Gy	24.3± 1.3	38.2 ±6.7	35.0±2.8	32.1±1.7	20.7±4.0
	Cur 20 µM+ 5Gy	20.8±3.2	19.9±4.6	30.9±3.1	21.2±1.1	17.8±3.7

Figure-11 Curcumin induced cytotoxicity on LNCaP cells

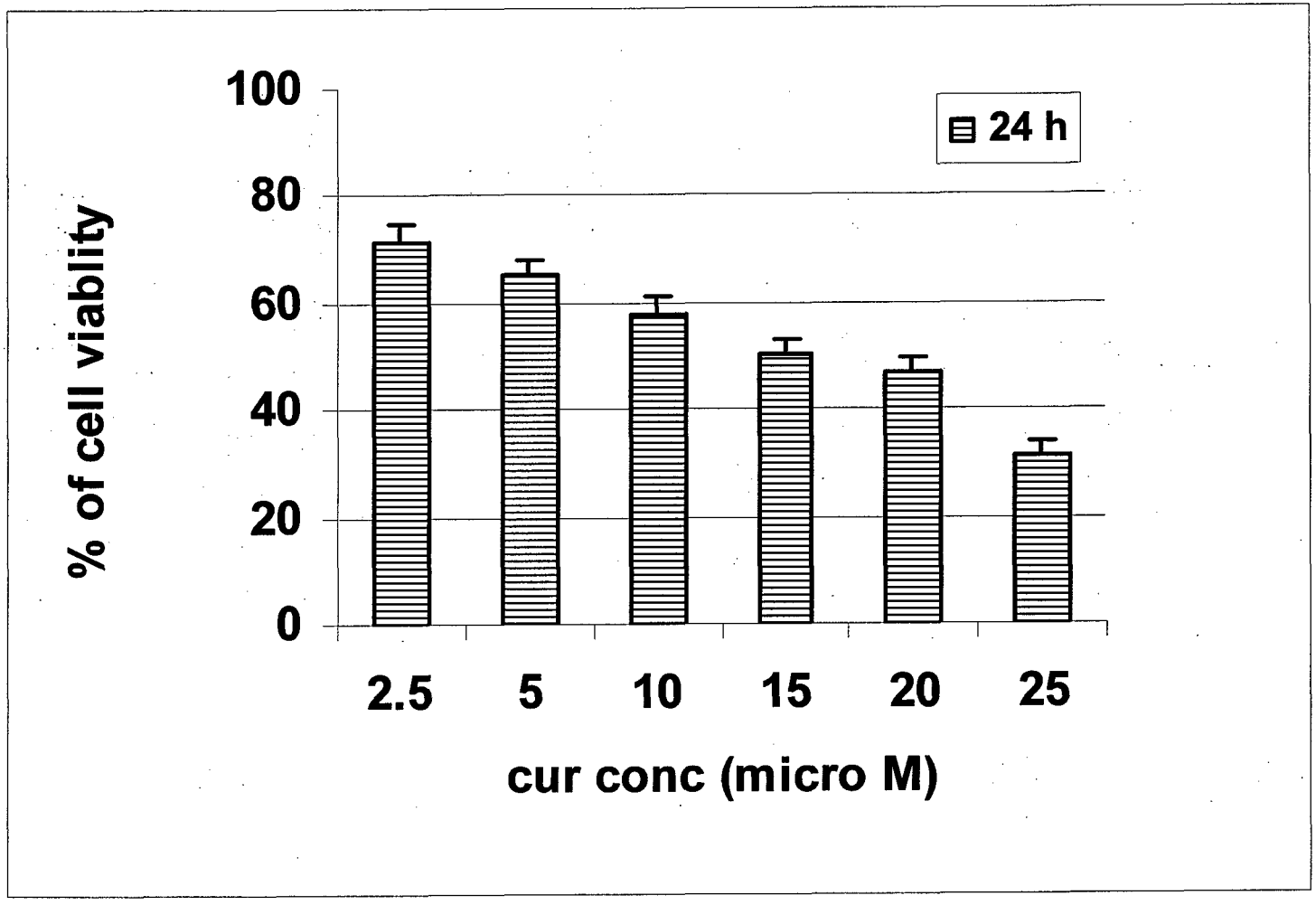


Figure-12 Cytotoxicity effects of curcumin and radiation on LNCaP cells.

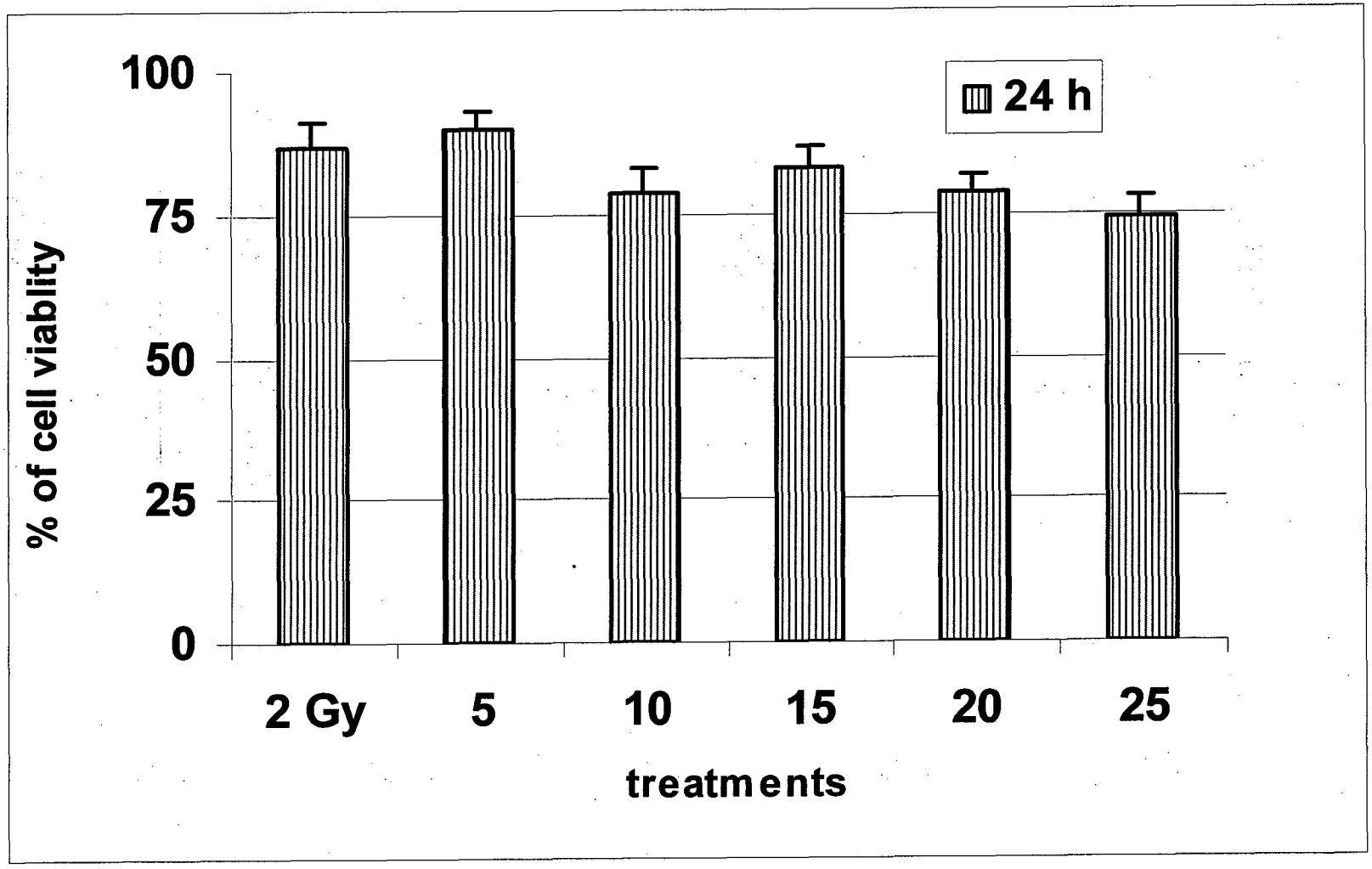


Figure 13 Curcumin and radiation induced apoptosis in LNCaP cells BY Annexin V-FITC

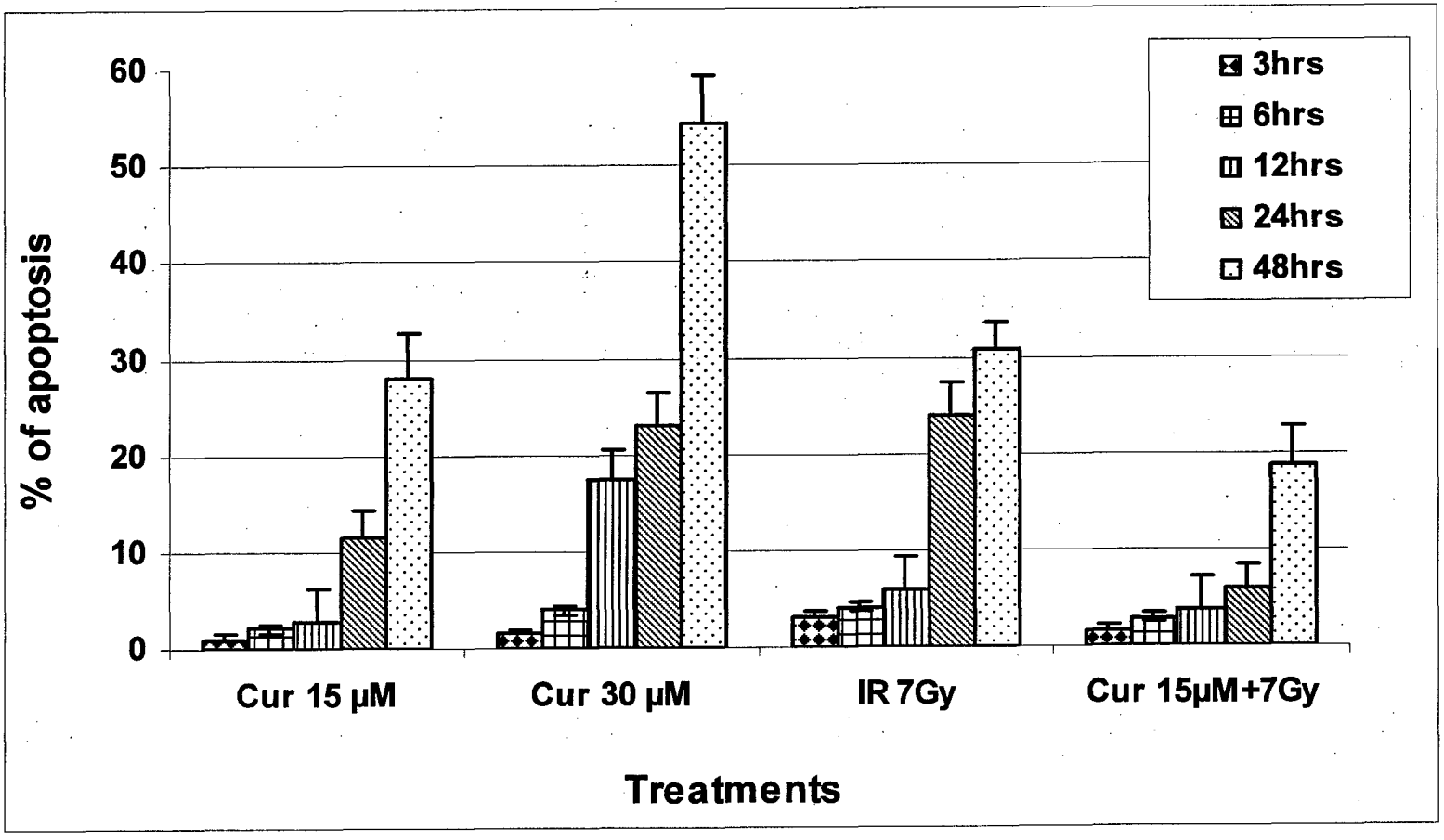


Figure-14 Curcumin and radiation induced apoptosis in LNCaP cells by TUNEL.

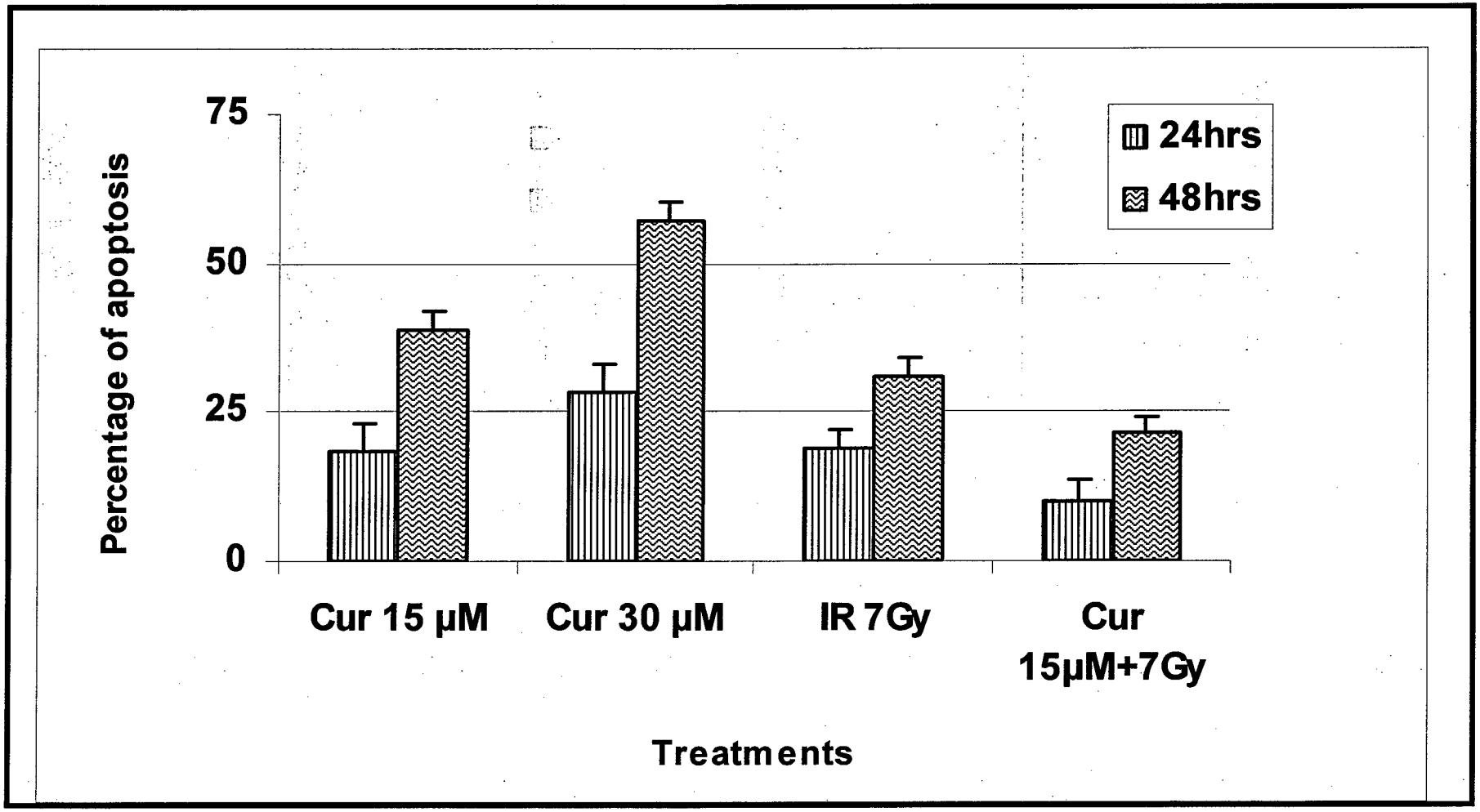
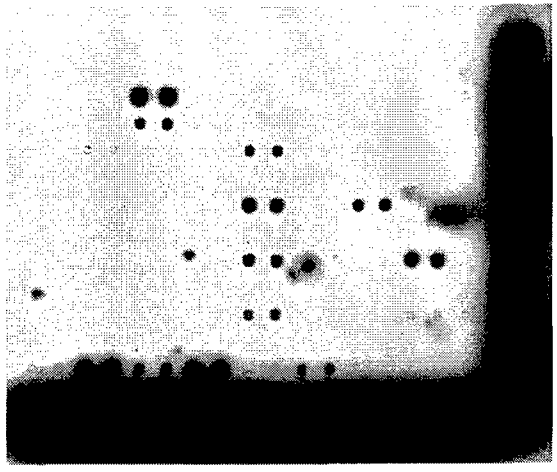


Table-5 Effects of curcumin or Ionizing radiation or in combination on cell cycle distribution in LNCaP cells.

Treatments		Time			
		6hrs	12hrs	24hrs	48hrs
G0/G1	UT	63.77± 3.78	58.39 ± 4.64	66.35 ± 7.01	63.51± 9.37
	Cur 15 µM	64.73± 4.21	65.79 ± 7.01	62.97 ± 6.76	73.51± 6.89
	IR 7Gy	57.13± 2.59	55.51± 6.23	73+ 7.24	87.95 ± 6.99
	Cur 10µM+7Gy	61.34± 1.09	56.93 ± 3.90	61.62+3.25	56.45 ± 6.12
S	UT	31.12 ±1.27	36.52 +_ 5.03	31.55+2.19	29.52 ± 3.90
	IR 7Gy	41.2 ±5.01	35.33+ 4.76	12.92 ± 1.09	11.35 ± 30
	Cur 15 µM	30.6 ± 2.08	28.99+1.99	36.82 ± 3.0	26.49 ± 2.18
	Cur 10µM+7Gy	32.4± 3.09	38.46+ 4.65	34.98 ± 3.01	37.56 ± 3 .01
G2/M	UT	5.12 ± 2.10	5.09 ± 1.98	2.1 ± 1.5	6.98 ± 2.67
	Cur 15 µM	4.67 ± 0.85	5.23±2.67	0.21± 0.01	0
	IR 7Gy	1.67 ± 1	9.16± 2.54	14.08 ± 2.19	0.7
	Cur 10µM+7Gy	6.26 ± 2.34	4.61 ±1.37	3.4 ± 1.02	5.99 ± 1.23

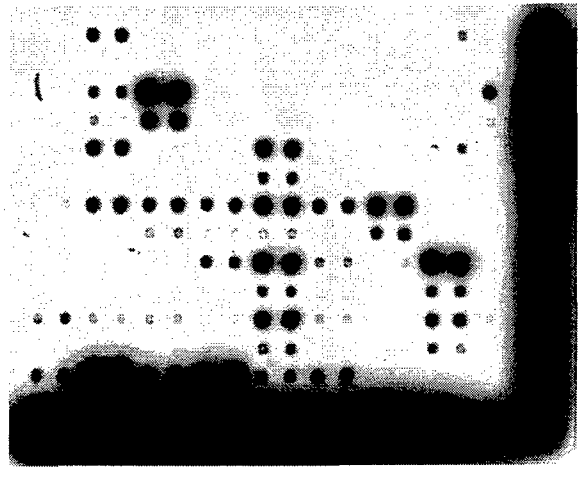
Fig 15. Du145 DNA/Protein micro-array

UT



CURCUMIN

IR



CURCUMIN+IR

Table-6 Schematic Diagram of the TranSignal Protein/DNA Array I

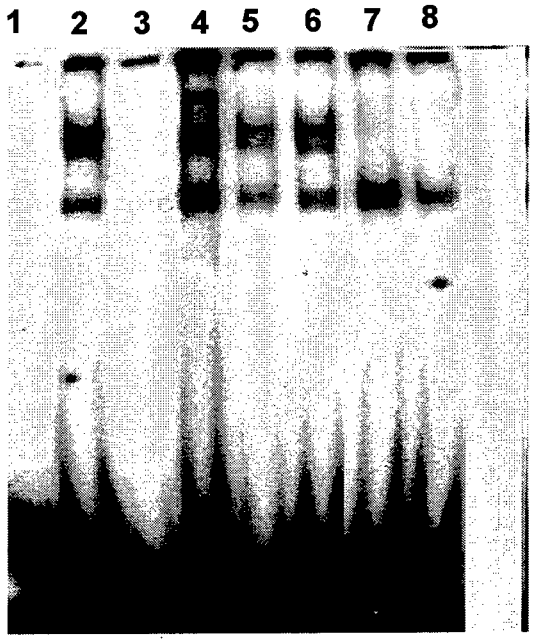
LEGEND

	1	2	
A	AP1	AP1	NORMAL
B	AP1	AP1	DILUTED 1:10

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
A	AP-1	AP-1	AP-2	AP-2	ARE	ARE	Brn-3	Brn-3	C/EBP	C/EBP	CBF	CBF	CDP	CDP	c-Myb	c-Myb	AP-1	AP-1	A
B	AP-1	AP-1	AP-2	AP-2	ARE	ARE	Brn-3	Brn-3	C/EBP	C/EBP	CBF	CBF	CDP	CDP	c-Myb	c-Myb	AP-1	AP-1	B
C	CREB	CREB	E2F1	E2F1	EGR	EGR	ERE	ERE	Ets	Ets	Ets1/PEA3	Ets1/PEA3	FAST-1	FAST-1	GAS/ISRE	GAS/ISRE	AP-2	AP-2	C
D	CREB	CREB	E2F1	E2F1	EGR	EGR	ERE	ERE	Ets	Ets	Ets1/PEA3	Ets1/PEA3	FAST-1	FAST-1	GAS/ISRE	GAS/ISRE	AP-2	AP-2	D
E	GATA	GATA	GRE	GRE	HNF-4	HNF-4	IRF-1	IRF-1	MEF-1	MEF-1	MEF-2	MEF-2	Myc-Max	Myc-Max	NF-1	NF-1			E
F	GATA	GATA	GRE	GRE	HNF-4	HNF-4	IRF-1	IRF-1	MEF-1	MEF-1	MEF-2	MEF-2	Myc-Max	Myc-Max	NF-1	NF-1			F
G	NFATc	NFATc	NF-E1	NF-E1	NF-E2	NF-E2	NFκB	NFκB	Oct-1	Oct-1	p53	p53	Pax-5	Pax-5	Pbx1	Pbx1			G
H	NFATc	NFATc	NF-E1	NF-E1	NF-E2	NF-E2	NFκB	NFκB	Oct-1	Oct-1	p53	p53	Pax-5	Pax-5	Pbx1	Pbx1			H
I	PPAR	PPAR	PPAR	PPAR	PPRE	PPRE	RAR(DR5)	RAR(DR5)	RXR(DR1)	RXR(DR1)	SIE	SIE	Smad SBE	Smad SBE	Smad 3/4	Smad 3/4			I
J	PPAR	PPAR	PPAR	PPAR	PPRE	PPRE	RAR(DR5)	RAR(DR5)	RXR(DR1)	RXR(DR1)	SIE	SIE	Smad SBE	Smad SBE	Smad 3/4	Smad 3/4			J
K	Sp1	Sp1	SRE	SRE	Stat1	Stat1	Stat3	Stat3	Stat4	Stat4	Stat5	Stat5	Stat6	Stat6	TFIID	TFIID			K
L	Sp1	Sp1	SRE	SRE	Stat1	Stat1	Stat3	Stat3	Stat4	Stat4	Stat5	Stat5	Stat6	Stat6	TFIID	TFIID			L
M	TR	TR	TR(DR4)	TR(DR4)	USF-1	USF-1	VDR(DR3)	VDR(DR3)	HSE	HSE	ARE	ARE							M
N	TR	TR	TR(DR4)	TR(DR4)	USF-1	USF-1	VDR (DR3)	VDR (DR3)	HSE	HSE	ARE	ARE							N
O																			O

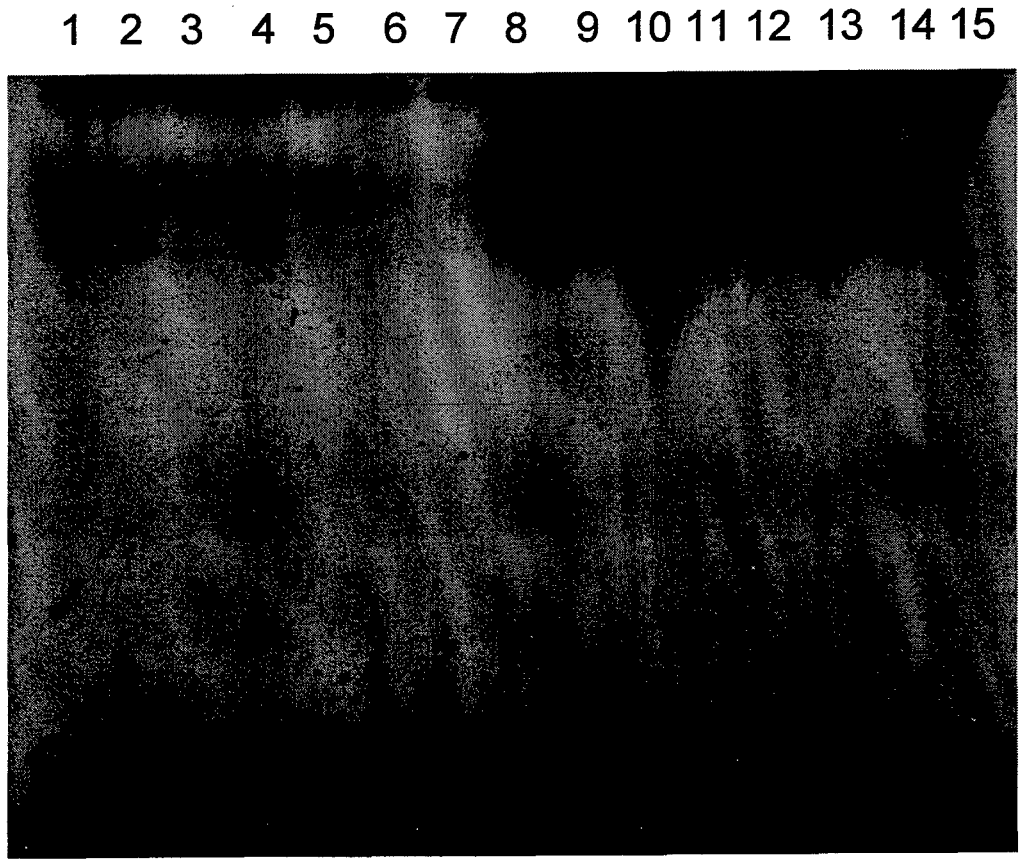
Figure 3. Schematic diagram of the TranSignal™ Protein/DNA Array I. The genes on the array are spotted in duplicate: the first row is DNA spotted normally, the second row is DNA diluted 1:10. The dark grey columns along the right and bottom sides of the array indicate where biotinylated DNA has been spotted. These spots are intended for alignment. (Note that the notch is at the top, right-hand corner.)

Fig.16 Curcumin down regulates radiation induced NFkβ AP 1 in PC-3 cells



1. probe alone
2. Positive control
3. Positive control + cold oligo
4. Positive control + anti-p65 antibody
5. PC-3 untreated
6. PC-3 + 5 Gy (3 hr)
7. PC-3 + 5 Gy + Cur (3 hrs)
8. PC-3 +5 Gy + Cur (6 hrs)

Fig.17 Curcumin down regulates radiation induced NFκβ AP 1 in DU-145 cells



1&8 – UT
2&9 curcumin 3hrs
3&10 curcumin 6hrs
4&11 IR-3hrs
5&12 IR-6hrs
6&13 Curcumin + IR 3hrs
7&14 Curcumin + IR 6hrs
15 Blank

Fig-18. Effect of curcumin on NFκβ & AP-1 in 22Rv1

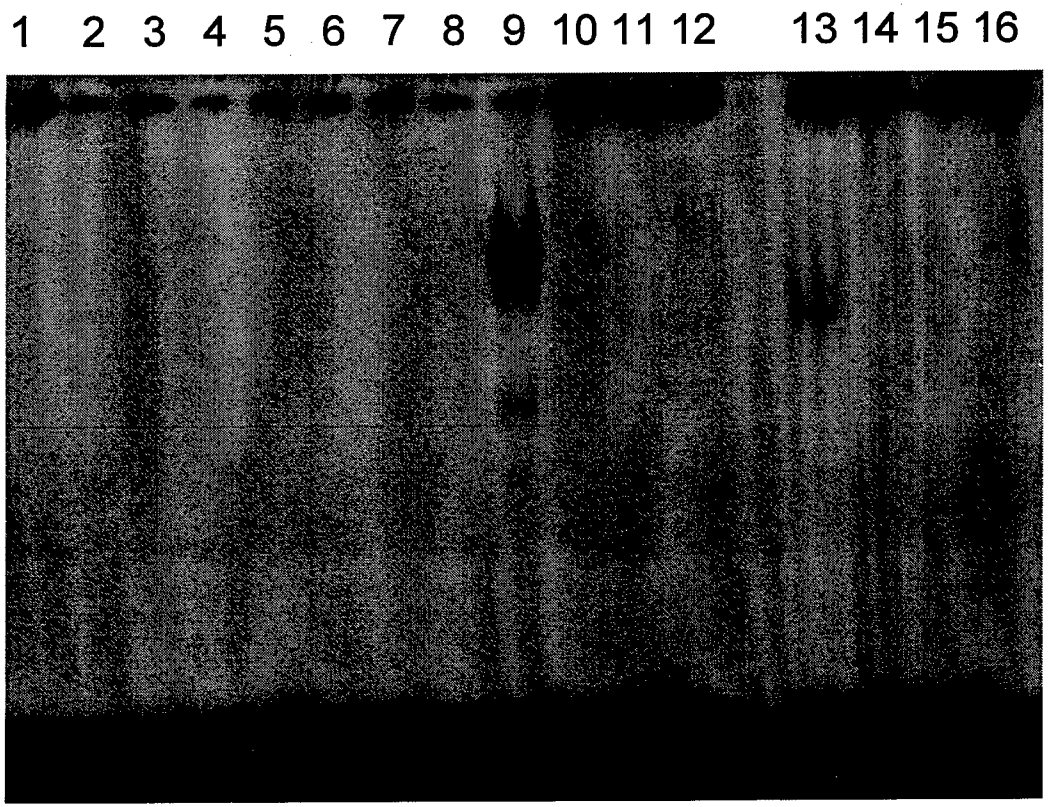
NFκβ **AP-1**

1&9 UT 4&12 IR-3hrs 7&15 Curcumin + IR 6hrs

2&10 curcumin 3hrs 5&13 IR-6hrs 8 Positive 16 Blank

3&11 curcumin 6hrs 6&14 Curcumin + IR 3hrs

Fig 19. Effect of curcumin on NFκβ & AP-1 in LNcaP



NFkβ **AP-1**

1 Blank 9 Positive
2&10 UT
3&11 curcumin 3hrs
4&12 Curcumin-6hrs
5&13 IR-3hrs
6&14 IR 6hrs
7&15 Curcumin + IR 3hrs
8&16 Curcumin + IR 6hrs

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Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3

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Curcumin (Diferuloylmethane) is a major chemical component of turmeric (*curcuma longa*) and is used as a spice to give a specific flavor and yellow color in Asian food. Curcumin exhibits growth inhibitory effects in a broad range of tumors as well as in TPA-induced skin tumors in mice. This study was undertaken to investigate the radiosensitizing effects of curcumin in p53 mutant prostate cancer cell line PC-3. Compared to cells that were irradiated alone ($SF_2 = 0.635$; $D_0 = 231$ cGy), curcumin at 2 and 4 μM concentrations in combination with radiation showed significant enhancement to radiation-induced clonogenic inhibition ($SF_2 = 0.224$: $D_0 = 97$ cGy and $SF_2 = 0.080$: $D_0 = 38$ cGy) and apoptosis. It has been reported that curcumin inhibits TNF-α-induced NFκB activity that is essential for Bcl-2 protein induction. In PC-3 cells, radiation upregulated TNF-α protein leading to an increase in NFκB activity resulting in the induction of Bcl-2 protein. However, curcumin in combination with radiation treated showed inhibition of TNF-α-mediated NFκB activity resulting in bcl-2 protein downregulation. Bax protein levels remained constant in these cells after radiation or curcumin plus radiation treatments. However, the downregulation of Bcl-2 and no changes in Bax protein levels in curcumin plus radiation-treated PC-3 cells, together, altered the Bcl2 : Bax ratio and this caused the enhanced radiosensitization effect. In addition, significant activation of cytochrome *c* and caspase-9 and -3 were observed in curcumin plus radiation treatments. Together, these mechanisms strongly suggest that the natural compound curcumin is a potent radiosesitizer, and it acts by overcoming the effects of radiation-induced prosurvival gene expression in prostate cancer.

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Keywords: prostate; curcumin; radiation; TNF-α; NFκB; Bcl-2; apoptosis

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Introduction

Prostate cancer is the cancer of second largest incidence among the male populations in the US, and the incidence has been increasing rapidly in the recent years (Greenlee *et al.*, 2000). According to a WHO report, 36% of the prostate cancer patients of the world, as in 2000, belong to US population (Wilkinson *et al.*, 2002). Prostate cancer cells are only modestly responsive or even unresponsive to the cytotoxic effects of chemotherapeutic agents or radiotherapy. Increased concentrations of cytotoxic drugs and higher dosages of irradiation fail to improve the response to therapy and it leads to resistance to apoptosis in prostate cancer cells. Thus, it is imperative to identify anticancer agents that are nontoxic and highly effective to induce cell death preferentially in tumor cells. Compounds occurring naturally in the human diet may be devoid of toxicity. Curcumin (Singh *et al.*, 1996) is a major chemical component of turmeric (Bhaumik *et al.*, 1999) and is used as a spice to give a specific flavor and yellow color in Asian food (Sharma, 1976). It is also used as a cosmetic as well as in some medical preparation. Curcumin has been reported to have several pharmacological effects including antitumor, anti-inflammatory and antioxidant properties (Sharma, 1976; Huang *et al.*, 1991). An epidemiological study revealed that low incidence of bowl cancer in Indians can be part in attributed to the presence of natural additives like curcumin in Indian cookery (Mohandas and Desai, 1999). Curcumin is inhibitory to a broad range of tumors such as mammary tumor, duodenal and colon cancer and TPA-induced skin tumors in mice (Huang *et al.*, 1998).

Curcumin has a potent role in inhibiting cellular migration and curcumin treatment of DU-145 cells suppressed the constitutive activation of both NF-κB and AP-1 (Mukhopoadhyay *et al.*, 2001). The molecular mechanism of NFκB inhibition by curcumin is unclear, but involved inhibition of IκB degradation (Jobin *et al.*, 1999; Mukhopadhyay *et al.*, 2001). In addition, the antiproliferaive activity of curcumin may also relate to its ability to block activation of RAS protein by inhibiting farnesyl protein transferase (Jiang *et al.*, 1996). Curcumin significantly inhibits prostate cancer growth (Dorai *et al.*, 2001) and has the potential to prevent the progression of this cancer to its

hormone-refractory state (Huang *et al.*, 1998). Curcumin induced apoptosis in both androgen-dependent and androgen-independent prostate cancer cells, by interfering with the signal transduction pathways and prevent the progression of the tumor to the hormone-refractory state (Singh and Aggarwal, 1995).

It has been shown that curcumin inhibits NFκB activation that in turn downregulates endogenous bcl-2 and bax_{xL} protein (Mukhopadhyay *et al.*, 2001). These observations lead us to hypothesize that curcumin can potentially inhibit radiation-induced prosurvival factors such as NFκB activation and Bcl-2 expression. To ascertain this hypothesis, we performed the experiments using androgen-independent, p53-null PC-3 cells. Our results show that curcumin in combination with radiation inhibits TNF-α-mediated NFκB activity, resulting in Bcl-2 protein downregulation in PC-3 cells. Also, curcumin enhanced radiation-induced apoptosis by releasing cytochrome *c* and activated caspases in combinations with radiation in PC-3 cells.

Results

Radiation-induced resistance through upregulation of prosurvival factors in prostate cancer cells

Resistance to radiation or chemotherapy may be due to interference of apoptotic pathways in cancer treatment. NFκB activation is thought to exert antiapoptotic effects in most cancer cells. In some cell types, the antiapoptotic effects of TNF-α appears to be mediated by the upregulation of NFκB activity (Beg and Baltimore, 1996; Wang *et al.*, 1996), resulting in induction of bcl-2 gene expression that causes resistance to treatments in cancer cells (Chendil *et al.*, 2002; Inayat *et al.*, 2002). Radiation caused an induction of TNF-α protein expression (Figure 1a), NFκB activity (Figure 1b) and Bcl-2 upregulation (Figure 1c) in PC-3 cells. The peak induction of TNF-α protein expression was observed at 48 h, and these results show that radiation induced prosurvival factors in p53-null prostate cancer cells.

Anti-TNF-α neutralizing antibody inhibits radiation-induced NFκB activity leading to repression of Bcl-2 protein

The protective responses of cells against radiation are DNA repair, activation of prosurvival transcription

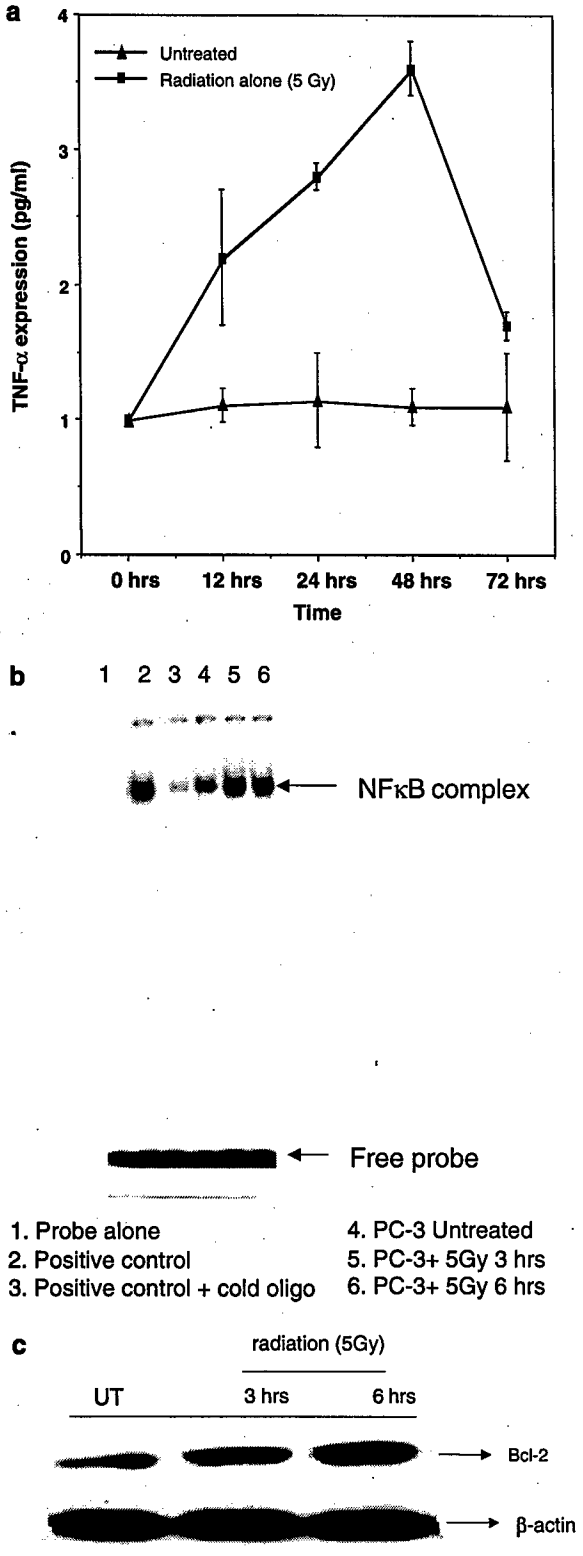

Figure 1 Ionizing radiation induces TNF-α protein, NFκB activity and Bcl-2 protein in PC-3 cells. (**a**) TNF-α protein estimation in a medium of untreated and irradiated PC-3 cells by enzyme-linked immunosorbent assay (ELISA). Error bars represent s.e.m. of two separate experiments performed in triplicate. (**b**) The effect of radiation on DNA binding activity of NFκB in PC-3 cells. EMSAs of NFκB binding complexes from PC-3 cells with 5 Gy of radiation. Lanes 2–5, positive control nuclear was used for appropriate experimental controls. Arrows indicate the position of super shift, NFκB complex band shift nonspecific binding and free probe. (**c**) Western blot analysis of Bcl-2 protein induction in PC-3 cells. Whole cell protein extracts were prepared from PC-3 cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the indicated time interval. The blot was probed with an antibody for Bcl-2 or β-actin

factors and induction of antiapoptotic genes. One of the prosurvival transcription factor, NFκB, is activated by ionizing radiation (Brach *et al.*, 1991). NFκB activates several downstream target genes such as Bcl-2 (Dixon *et al.*, 1997; Tamatani *et al.*, 1999), which is responsible for the protection of cells against radiation-induced apoptosis. The activation of NFκB by radiation also depends on radiation-induced TNF-α protein (Baldwin, 1996; Bierhaus *et al.*, 1997). Moreover, NFκB is known to be a cell survival and antiapoptotic molecule (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996). To test whether TNF-α induction by radiation is necessary for NFκB activation and Bcl-2 upregulation, we performed Western blot analysis of cells either left untreated or treated with anti-TNF-α neutralizing antibody plus radiation. As shown in Figure 2a, DNA binding activity of NFκB increased approximately sixfolds in these cells treated with recombinant TNF-α protein. Whereas, the radiation-induced NFκB was inhibited when PC-3 cells were exposed to anti-TNF-α neutralizing antibody (Figure 2a). Similarly, recombinant TNF-α protein induced Bcl-2 expression in these cells while anti-TNF-α neutralizing antibody repressed the radiation-induced Bcl-2 expression (Figure 2b). These results suggest that radiation-induced NFκB activity depends on radiation-induced expression of TNF-α. Altogether, these observations indicate that radiation induces TNF-α that in turn triggers NFκB activation and Bcl-2 induction in PC-3 cells.

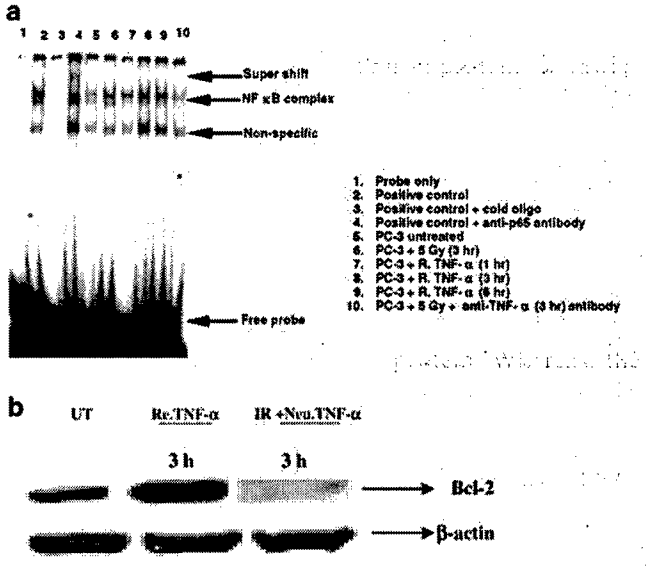

Figure 2 Neutralizing antibody to TNF-α or AD5-IκB super-repressor inhibits radiation-induced NFκB activity leading to repression of Bcl-2 protein in PC-3 cells. (**a**) EMSAs of NFκB binding complexes from PC-3 cells were left untreated or treated with recombinant TNF-α and/or 5 Gy of radiation with neutralizing anti-TNF-α antibody. Nuclear cell extracts (5 μg) from untreated or irradiated cells were incubated with 32p-labeled NFκB DNA probe, followed by analysis of DNA binding activities. Arrows indicated the position of NFκB complex bindings. (**b**) Whole cell protein extracts were prepared from PC-3 cells that were left untreated (UT) or treated with recombinant TNF-α and/or irradiated at 5 Gy and incubated for the indicated time interval. The blot was probed with an antibody for Bcl-2 or β-actin

Curcumin inhibits radiation-induced prosurvival factors in PC-3 cells

Previously, it has been shown that curcumin inhibits TNF-α-mediated NFκB activation. In addition, curcumin downregulates endogenous bcl-2 and bax_{xL} protein level (Mukhopadhyay *et al.*, 2001). In order to inhibit the radiation-induced antiapoptotic function in cells, we treated PC-3 cells either with curcumin or radiation alone or in combination. Interestingly, curcumin inhibited radiation-induced TNF-α protein expression (Figure 3a) and resulted in downregulation of NFκB activation (Figure 3b) and Bcl-2 protein expression (Figure 3c). Together, these data strongly suggest that curcumin is a potent inhibitor of radiation-induced prosurvival factors in PC-3 cells.

Curcumin enhance radiation-induced clonogenic inhibition in PC-3 cells

PC-3 cells conferred enhanced resistance to radiation since curcumin inhibits radiation-induced prosurvival factors such as NFκB and Bcl-2. Effects of curcumin either alone or in combination with radiation on cell survival studied with colony-forming assay. Curcumin enhanced significantly the radiation-induced clonogenic inhibition ($SF_2 = 0.224$: $D_0 = 97$ cGy and $SF_2 = 0.080$: $D_0 = 38$ cGy at 2 and 4 μM concentrations) compared to cells treated with curcumin alone (Figure 4a) or radiation alone ($SF_2 = 0.635$; $D_0 = 231$ cGy) (Figure 4b). Interestingly, a significant enhancement in the radiosensitizing effect of curcumin was observed at 2 and 4 μM concentrations. These results indicate that the natural compound curcumin inhibited the growth of PC-3 cells and significantly enhanced the effect of radiation (Table 1).

Curcumin enhances radiation-induced apoptosis in PC-3 cells

Terminal transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining was performed with or without curcumin/radiation after 24 and 48 h of treatment to determine the induction of apoptosis. By TUNEL assay, the incidence of apoptosis after 24 and 48 h of radiation over the untreated population was 2.61 and 4.88% compared to curcumin alone treated cells (7.23 and 11.56%, respectively). The combination of curcumin and radiation significantly enhanced induction of apoptosis in these cells after 24 h (21.39%) and 48 h (27.57%) of treatment (Figure 5a). By flow cytometry assay, using MC-540 and Hoechst 342 staining, the incidence of apoptosis after 24 and 48 h of radiation over the untreated population was 3.81 and 6.25%, compared to curcumin alone treated cells (9.6 and 13.22%, respectively). However, with combination of radiation and curcumin the incidence of apoptosis after 24 and 48 h was 18.31 and 29.90%, respectively

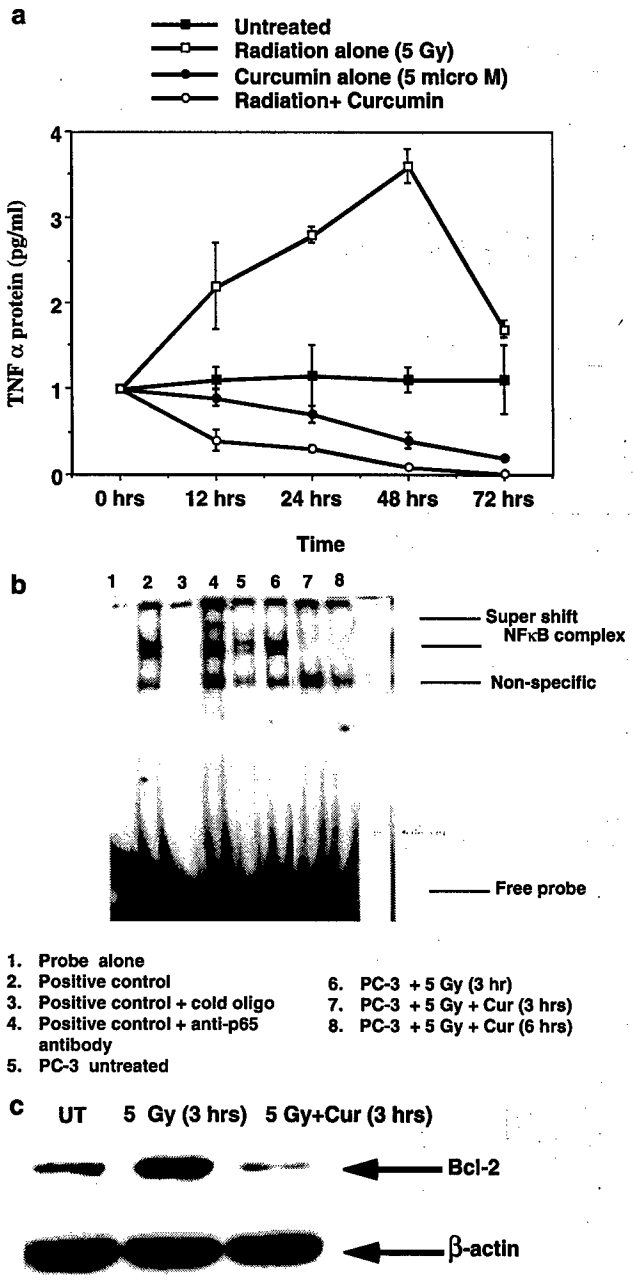

Figure 3 Curcumin downregulates radiation-induced NFκB activity and Bcl-2 protein expression. (**a**) TNF-α protein estimation in a medium of untreated/ftreated with curcumin (5 μM) or radiation (5 Gy) or in combination (curcumin 2 μM plus radiation (5 Gy)) of PC-3 cells by ELISA. Error bars represent s.e.m. of two separate experiments performed in triplicate. (**b**) The effect of Curcumin on radiation-induced NFκB activity in PC-3 cells. Arrows indicated the position of super shift, NFκB complex, nonspecific binding and free probes. (**c**) Western blot analysis of Bcl-2 expression on PC-3 treated with curcumin with or without radiation. Whole cell protein extracts were prepared from PC-3 cells that were left untreated (UT) or treated with or with out curcumin or exposed to a 5 Gy dose. The blot was probed with antibodies for Bcl-2, or β-actin

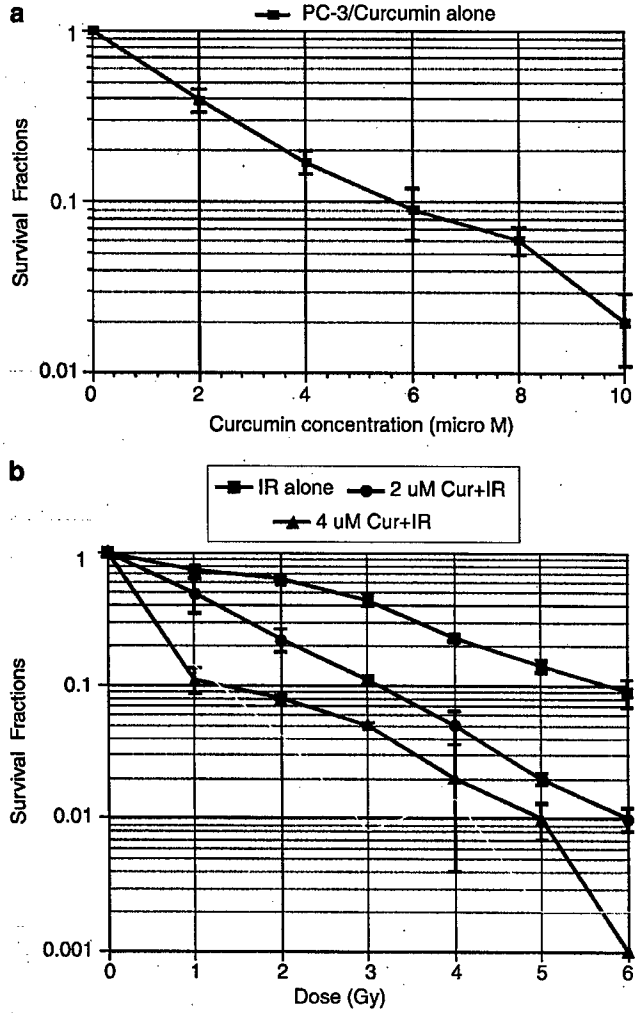

Figure 4 Curcumin enhance radiation-induced clonogenic inhibition in PC-3 cells: (**a**) Curcumin alone or (**b**) radiation or in combination with 2 or 4 μM concentration of curcumin induced clonogenic inhibition of PC-3 cells. Cell survival curve of PC-3 cells with treatments as assayed by colony-forming ability and analysed by Single Hit Multi-Target (SHMT) model curve fit. The data shown are representative of the combined mean of two independent experiments

(Figure 5b). Thus, these results demonstrate that curcumin significantly enhanced the radiation-induced apoptosis in PC-3 cells.

Curcumin downregulates radiation-induced Bcl-2 protein expression in PC-3 cells

After confirming that curcumin inhibits radiation-induced prosurvival factors in PC-3 cells, we performed Western blot analysis for Bcl2 and Bax protein expression after treating PC-3 cells with curcumin or radiation or in combination. Bax and Bcl-2 are two discrete members of the gene family involved in the regulation of cellular apoptosis. Interestingly, no change in the level of bax protein was observed after the combined treatment (Figure 6). Since curcumin down-regulates radiation-induced Bcl-2 and no changes in the

Table 1 Radiation inactivation estimates of PC-3 cells treated with curcumin or radiation alone or in combination

Treatment	SF_2	SF_2 *ER*	D_0 *(Gy)*	D_0 *ER*	*ER*
Radiation alone	0.635	—	231 cGy	—	
IR + 2 μM curcumin	0.224	2.83	97 cGy	2.38	5.21
IR + 4 μM curcumin	0.080	7.93	38 cGy	6.07	14

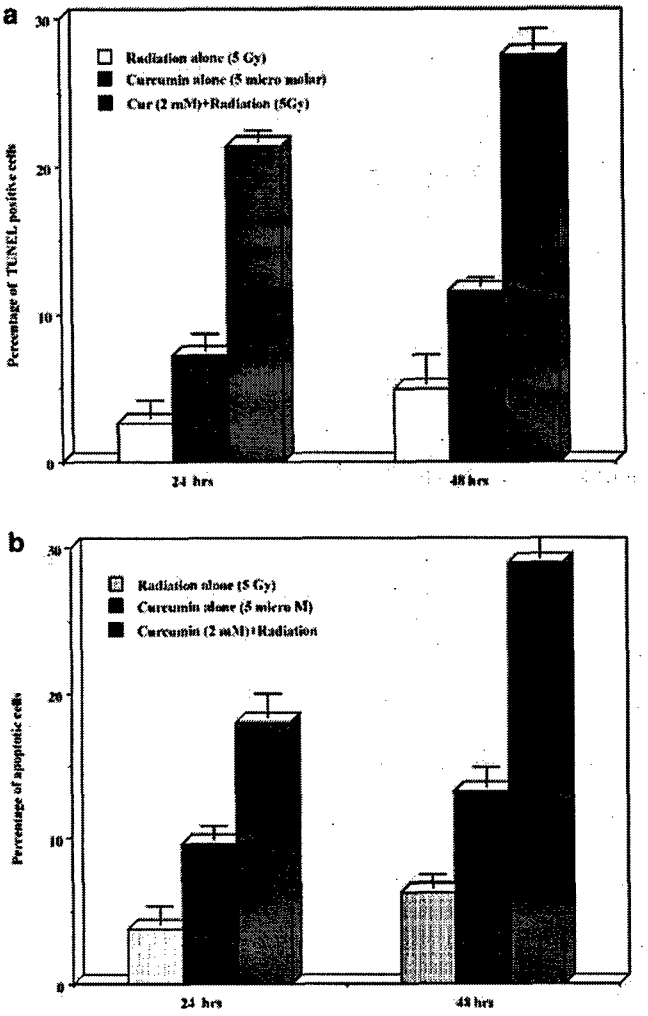

Figure 5 Curcumin enhances radiation-induced apoptosis in PC-3 cells: (a) Quantification of apoptosis by TUNEL method. (b) Quantification of apoptosis in by Hoechst 33342 (Ho342) and merocyanin 540 (MC540) staining. The error bar represents s.e. and the data shown are representative of the combined mean from two independent experiments

bax protein levels were observed, Bcl2:Bax ratio changed and it may have caused the induction of apoptosis in PC-3 cells.

Curcumin induced cytochrome c *release and caspases activation in PC-3 cells*

To confirm the involvement of the mitochondrial pathway of apoptosis, we analysed the activation of

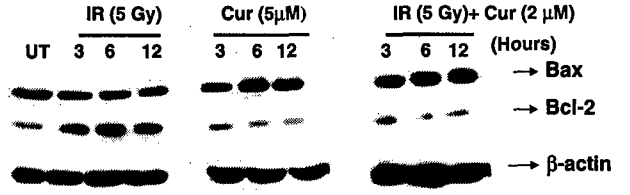

Figure 6 Curcumin downregulated radiation-induced Bcl-2 protein in PC-3 cells: Whole cell protein extracts were prepared from PC-3 cells that were left untreated or treated with radiation or curcumin or in combination and incubated for the indicated time interval. The blot was probed with antibodies for Bcl-2, Bax and β-actin

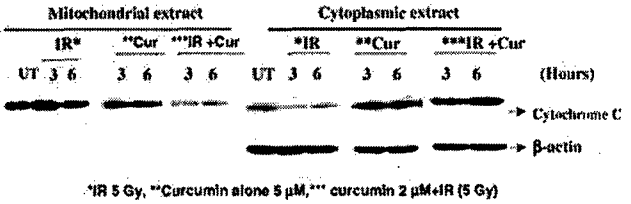

Figure 7 Curcumin induced cytochrome *c* release in PC-3 cells: Cytochrome *c* was assessed in untreated, treated with radiation or curcumin or in combination after 3 and 6 h in PC-3 cells, and cytosol and mitochondria extracts were extracted. Western blot analysis was used to determine the cytochrome *c* expression in both cytosol and mitochondria extracts. Loading control β-actin antibody was used for cytosol extracts

cytochrome *c* release from the mitochondria by Western blot analysis. It is known that cytochrome *c* releases from mitochondria into the cytosol and binds to the apoptotic protease activating factor (Apaf) complex and triggers the activation of procaspase-9 to the active caspase-9 (Reed, 1997). As shown in Figure 7, a marked fraction of the cytochrome *c* was released from the mitochondria, of curcumin and in combination with radiation-treated cells at 3 h, and the release was more pronounced at 6 h.

Caspases play a pivotal role in the execution of programmed cell death (Janicke *et al.*, 1998; Juo *et al.*, 1998; Kuida *et al.*, 1998; Earnshaw *et al.*, 1999), and in particular, we evaluated caspase-9 activity because it represents the apical caspase of the mitochondrial (intrinsic) pathway (Kuida *et al.*, 1998). Caspase-3 activation has been shown to be one of the most important cell executioners for apoptosis (Janicke *et al.*, 1998). A marked time-dependent increase in the activities of caspase-9 and -3 was observed in cells treated with 5 μM curcumin or in combination of curcumin (2 μM) with radiation. However, the sequential pattern of activation of these caspases was markedly different. There was significant activation of caspase-9 as early as 3 h of treatment, and the activity continued to increase till 24 h of the assay (Figure 8a). On the other hand, significant activation of caspase-3 activity was seen at 12 h after treatment (Figure 8b). The increase in the release of cytochrome *c* was in agreement with the data showing the continued increase in caspase-9 activity after 3 h treatment. The close association of the release of cytochrome *c* from mitochondria with the

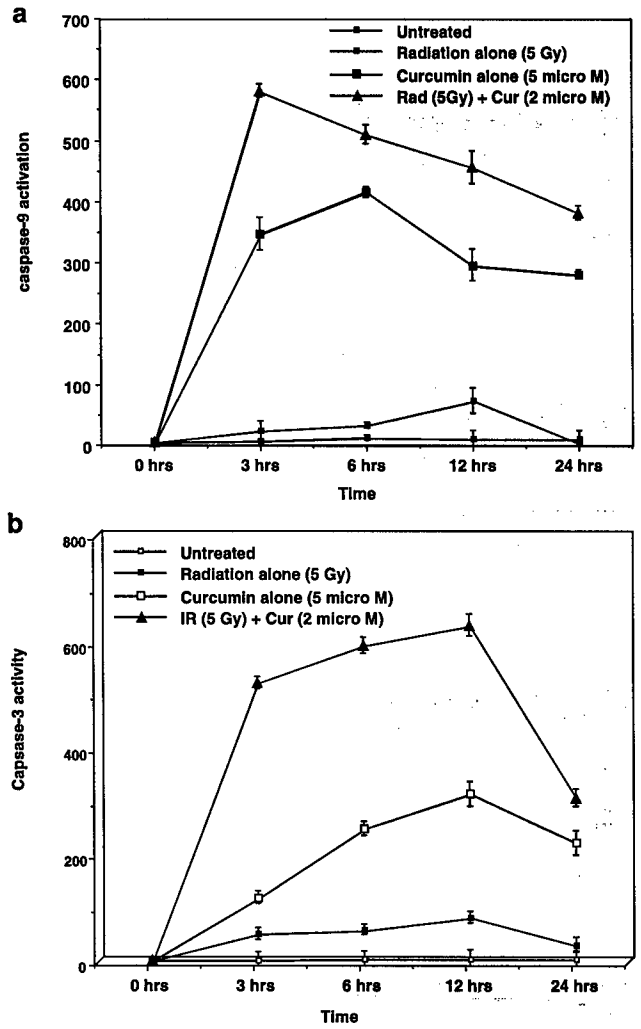

Figure 8 Curcumin induce caspase-3 and caspase-9 activation in PC-3 cells: PC-3 cells were left untreated or treated with radiation or curcumin or in combination and and cell lysates prepared for time interval indicated. The enzymatic activity of cell lysates towards tetrapeptide chromogenic substrates, (**a**) DEVD-pNA (for caspase 3-like) and (**b**) LEHD-pNA (for caspase 9) was determined. Caspase activities are expressed as fold change of control and presented as mean ± SE of two samples

concurrent increase in caspase-9 provide evidence that curcumin induces apoptosis in PC-3 cells through the mitochondrial pathway.

Curcumin radiosensitizes PC-3 cells by inducing G_2/M block of cell cycle

To understand the mechanism that curcumin causes enhanced radiation-induced clonogenic inhibition, we performed flow cytometry to analyse the cell cycle changes induced by radiation in PC-3 cells. Curcumin caused a strong G_2/M block, which is an important phase sensitive to radiation. Curcumin-treated cells showed 32.55% of G_2/M block at 12 h, 48.10% at 24 h, 42.75% at 48 h and 35.89% at 72 h(Table 2). Thus, the G_2 block in curcumin-treated cells when combined with radiation caused enhanced radiosensitization, whereas radiation- or curcumin-treated cells showed no significant G_2/M block.

Discussion

Our studies showed that radiation induces prosurvival factors such as increased NFκB activity and Bcl-2 upregulation in PC-3 cells. Ionizing radiation induce NFκB activation (Hallahan *et al.*, 1989; Van Antwerp *et al.*, 1996) and it plays an important role in inhibiting TNF-α or chemotherapy-induced apoptosis (Wang *et al.*, 1996; Plummer *et al.*, 1999). TNF-α is also a potent inducer of NFκB activity (Hallahan *et al.*, 1989; Van Antwerp *et al.*, 1996). The multiplicity of mechanisms of NFκB activation and its role in inhibition of antiapoptotic function is more complex. The antiapoptotic target genes for NFκB includes Bcl-2 (Tamatani *et al.*, 1999), Bcl-xL (Dixon *et al.*, 1997; Tamatani *et al.*, 1999) and Bcl-2 homologue A1/Bfl-1(Wang *et al.*, 1999). We reported that ectopic overexpression of Bcl-2 in prostate cancer cells showed enhanced radiation resistance and inhibition of apoptosis in prostate cancer cells and other tumor cell types (Hockenbery *et al.*, 1990; Sentman *et al.*, 1991). Induction of prosurvival and antiapoptotic genes

Table 2 Effects of curcumin or ionizing radiation or in combination with curcumin and radiation on cell cycle distribution

Cell cycle	*Treatments*	*12 h*	*24 h*	*48 h*
G0/G1	Untreated	55.45±2.21	56.53±4.21	53.23±4.28
	Curcumin alone	28.45±3.43	18.65±3.76	20.05±1.92
	Radiation alone	15.62±2.34	21.61±1.72	36.11±2.69
	IR+Cur	24.21±3.99	25.22±2.24	20.85±1.65
S	Untreated	24.21±3.05	21.32±5.11	28.39±3.76
	Curcumin alone	39.00±1.98	33.25±1.07	37.20±4.56
	Radiation alone	35.47±2.98	13.25±1.09	22.86±3.30
	IR+Cur	48.32±3.12	44.20±5.12	54.65±5.74
G2-M	Untreated	20.34±3.67	22.15±1.98	18.38±2.01
	Curcumin alone	32.55±3.10	48.10±2.16	42.75±3.21
	Radiation alone	48.82±2.73	65.14±5.92	41.03±4.19
	IR+Cur	27.48±2.12	30.58±4.21	24.50±1.99

strongly suggests that PC-3 cells harbor a tight regulatory loop that inhibits the cell killing effects of ionizing radiation.

Curcumin has been reported to be a potent antiproliferative agent for many tumor types (Rao *et al.*, 1995; Sikora *et al.*, 1997) and it acts as a proapoptotic agent in a variety of cancer cell lines (Kuo *et al.*, 1996; Khar *et al.*, 1999). Exposure of PC-3 cells to curcumin inhibited radiation-induced Bcl-2 expression, indicating that radiation-induced TNF-α is necessary to activate NFκB, which is required for the induction of Bcl-2 protein. This is the first report showing that curcumin inhibits endogenous TNF-α as well as radiation-induced TNF-α protein expression in PC-3 cells. Inhibitory effects of curcumin on NFκB activation have been documented in prostate cancer cells (Mukhopadhyay *et al.*, 2001), mouse fibroblast cells (Huang *et al.*, 1991), human leukemia cells (Singh and Aggarwal, 1995) and human colon epithelial cells (Plummer *et al.*, 1999). Curcumin inhibits NFκB activation by inhibiting IκBα phosphorylation that is necessary to export NFκB from cytosol to nucleus and to activate its target genes. However, this is the first documented report demonstrating that curcumin is a potent radiosenstizer in prostate cancer cells and this sensitization is conferred by the inhibition of radiation-induced prosurvival factors such as NFκB and Bcl-2. Hence, the downregulation of endogenous and radiation-induced bcl-2 protein expressions in PC-3 cells will have significant therapeutic benefits in a majority of prostate cancer patients, since bcl-2 protein is overexpressed in these patients. Curcumin also inhibits cell proliferation induced by growth factors. Correlation between inhibition of cell proliferation and different phases of cell cycle by curcumin has been reported in the literature (Chen and Huang, 1998; Chen *et al.*, 1999). Curcumin induces cell cycle arrest in G2/M phase in breast cancer cells. These findings well correlated with our results since curcumin-treated cells showed G2/M arrest of cell cycle, which is sensitive to radiation and therefore this leads to enhanced radiosensitization.

Activated protein (AP-1) is a transcription factor activated by UV radiation, phorbol ester and asbestosis (Shaulian and Karin, 2002). AP-1 promotes several cellular genes that are responsible for cell proliferation and also transformation of preneoplastic to neoplastic state (Dong *et al.*, 1995). Several reports show that curcumin suppresses AP-1 activation; in our study also curcumin inhibits endogenous and radiation-induced AP-1 in PC-3 cells (data not shown).

We found that curcumin induced apoptosis by the activation of the downstream caspase-9, which has been shown to play an important role in apoptosis induced by several conditions (Ohta *et al.*, 1997; Mow *et al.*, 2001). In this study, caspase-9 activation was preceded by the activation of caspase-3, the apical caspase of the intrinsic mitochondrial pathway of apoptosis. Similarly, curcumin induced the release of cytochrome *c* into the cytosol after 3 h, and this release markedly increased after 6 h of treatment. Our results show curcumin downregulates radiation-induced bcl-2 protein expression that suggests that this protein is involved in the release of cytochrome *c* from mitochondria.

In recent clinical trails, curcumin was given a dosage of 8000 mg/day, and the peak serum concentration of $1.77 \pm 1.87\,\mu$M after 2 h of intake of curcumin has been reported (Cheng *et al.*, 2001). In our results, a significant enhancement of radiosensitizing effect was observed at 2 and 4 μM concentrations of curcumin by colony-forming assays. Hence, it is possible to achieve 2 μM concentration of curcumin in the serum concentration by consumption of 8000 mg/day of curcumin and this dose of curcumin in the serum will enhance the radiation effect in prostate cancer patients.

Apoptotic assays indicate that radiation caused significantly enhanced apoptosis in curcumin-treated cells. These results indicate that the natural compound, curcumin, at nontoxic doses inhibited the growth and induced apoptosis in PC3 cells and significantly enhanced the effect of radiation. It has been shown that curcumin induce apoptosis either by mitochondrial-dependent or mitochondrial-independent mechanism depending on the cell types. Curcumin-induced mitochondrial-independent apoptosis has been shown in breast cancer cell lines (Mehta *et al.*, 1997), basal cell carcinomas (Jee *et al.*, 1998) and T-Jurkat cells (Piwocka *et al.*, 1999).

In conclusion, curcumin, a major active component of turmeric, has been reported to induce growth inhibition and induce apoptosis in many cancer cell types. In this study we, for the first time, report that curcumin is a potent radiosensitizer that inhibits growth of human prostate PC-3 cancer cells and downregulates radiation-induced prosurvival factors and enhance radiation-induced sensitivity in PC-3 cells.

Materials and methods

Cell line

Human prostate cancer cells (PC-3 cells) were obtained from the American Type Culture Collection and maintained as adherent monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Curcumin

Curcumin (99% purity) (E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was purchased from Sigma Chemical Co. (St Louis, MO, USA) and stored as 100 mM stock solution in DMSO, protected from light at -20°C. At 24 h after plating the cells, the medium was removed and replaced with fresh medium containing DMSO or medium containing different concentrations of curcumin in RPMI medium. For treatments, cells were left untreated or treated with radiation (5 Gy) alone or curcumin (5 μM) alone or in combination of curcumin with radiation. For combination treatment, curcumin (2 μM) was added to the cultures 2 h prior to radiation (5 Gy).

Irradiation

A 100 kV industrial X-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose

rate with a 2 mm Al plus 1 mm Be filter was ~2.64 Gy/min at a focus-surface distance of 10 cm.

Western blot analysis

Total protein extracts from untreated cells or cells treated with curcumin alone or irradiated alone or in combination (curcumin plus radiation) at various time intervals were subjected to Western blot analysis as described (Chendil *et al.*, 2002) using Bcl-2 monoclonal antibody (sc-509) (Santa Cruz, CA, USA), Bax monoclonal antibody (sc-493) (Santa Cruz, CA, USA), cytochrom *c* monoclonal antibody (Biovision, Inc., CA, USA), or for loading control the β-actin antibody (Sigma Chemical Co, St Louis, MO, USA) were detected using the chemiluminescence method.

Colony-forming assay

For clonogenic cell survival studies, two different cell concentrations in quadruplet sets were used for each treatment. Cell lines were left untreated or exposed to 0.5–6 Gy dose of radiation or treated with various concentration of curcumin or for combinatation treatment curcumin (2 μM or 4 μM) was added to the cultures 2 h prior to radiation (5 Gy). After incubation for 10 or more days, each flask was stained with crystal violet and the colonies containing more than 50 cells were counted. The surviving fraction (SF) was calculated as a ratio of the number of colonies formed and the product of the number of cells plated and the plating efficiency. The curve was plotted using *X–Y* log scatter (Delta Graph®4.0), and by using the formula of the SHMT model, the D_0 was calculated. D_0 is the dose required for reducing the fraction of cells to 37%, indicative of single-event killing. SF_2 is the survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy.

Flow cytometry

Flow cytometry was performed as described earlier (Chendil *et al.*, 2002). Untreated and treated cells (1×10^6) were washed in phosphate-buffered saline (PBS) and fixed in ice-cold ethanol. Fixed cells were pelleted and resuspended in 500 μl of PBS. RNA was eliminated by treating cells with RNAse A (Sigma, St. Louis, MO, USA). Then, the cells were stained by propidium iodide in PBS and analysed for cell cycle phases by flow cytometry, a FACStar calibur (Becton Dickinson) cell sorter.

Quantification of apoptosis

Apoptosis was quantified by TUNEL staining and flow cytometry. The ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA), which detects DNA strand breaks by TUNEL, was used as described (Ahmed *et al.*, 1996). Briefly, cells were seeded in chamber slides and the next day the cells were left untreated or treated with curcumin or radiation or in combination. After 24 and 48 h, the DNA was tailed with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimen was counterstained with propidium iodide and antifade. The stained specimen was observed in triple band-pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells showing apoptosis, four experiments in total were performed, and approximately 1000 cells were counted in each experiment. For flow cytometry, cells were lifted by using nonenzymatic cell dissociation medium (Sigma) and washed with PBS and stained with Hoechst (Ho342) and merocyanine (MC540) and analysed by flow cytometry using a FACStar Plus cell sorter as described (Ahmed *et al.*, 1996).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from untreated and treated cells and EMSA was preformed as described previously (Chendil *et al.*, 2002) with some modification. Briefly, cells were scraped and washed with cold PBS and repelleted. The pellet was suspended in 1 ml of Icecold buffer A (10 mM HEPES pH 7.8, 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 10 μg/ml of aprotinin, 0.5 μg/ml leupeptin, 3 mM PMSF and 3 mM dithiothreitol) for 5 min on ice. The crude nuclei were pelleted by centrifugation for 5 min. The crude nuclei pellet was suspended in 50 μl of buffer B (10 mM HEPES, pH 7.8, 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 10 μg/ml of aprotinin, 0.5 μg/ml leupeptin, 3 mM PMSF, 3 mM dithiothreitol and 10% NP-40). After 20 min of incubation on ice, the suspension was centrifuged, and supernatant was collected. Equal volume of cold buffer C (50 mM HEPES, pH 7.4, 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 3 mM PMSF, 3 mM DTT and 10% (v/v) glycerol) was added to the supernatant and incubated on ice for 5 min with intermittent vortexing. The extracts were then centrifuged for 10 min and the supernatant was divided into aliquots and frozen at –80°C.

Analysis of DNA binding by EMSA was performed using 2 mg of poly(dI-dC) (Sigma Chemical Co, St Louis, MI, USA) as nonspecific competitor DNA. The binding reactions contained 10 000 c.p.m. of ^{32}P-labeled double-stranded oligonucleotide probe with a high affinity for NFκB binding (Promega, Madison, WI, USA). For supershift experiments, anti-p65 antibody was incubated with binding buffer and nuclear extract for 1 h prior to adding the oligo probe. Binding reactions were electrophoresed on a 4% PAGE in 0.5 × TBE buffer to separate the bound and unbound probe.

Caspase activity

To measure the activity of caspases-3 and -9 in PC-3 cells, a fluorimetric assay was used according to the instruction of the manufacturer (Biovision, CA, USA). Briefly, cells were left untreated and treated either with curcumin or radiation or in combination. Cells were collected and resuspended in cold lysis buffer and incubated for 10 min on ice. In all, 50 μl of 2 × reaction buffer wad added and incubated for 2 h at 37°C with fluorogenic substrates, DEVD-AFC (caspase-3) and LEHD-AFC (caspase-9) in a reaction buffer. The release of fluorocrome AFC was measured at 400 nm excitation and 505 nm emissions using a fluorescence spectrophotometer.

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References

Ahmed MM, Venkatasubbarao K, Fruitwala SM, Muthukkumar S, Wood Jr DP, Sells SF, Mohiuddin M and Rangnekar VM. (1996). *J. Biol. Chem.*, **271**, 29231–29237.
Baldwin Jr AS. (1996). *Annu. Rev. Immunol.*, **14**, 649–683.
Beg AA and Baltimore D. (1996). *Science*, **274**, 782–784.
Bhaumik S, Anjum R, Rangaraj N, Pardhasaradhi BV and Khar A. (1999). *FEBS Lett.*, **456**, 311–314.
Bierhaus A, Zhang Y, Quehenberger P, Luther T, Haase M, Muller M, Mackman N, Ziegler R and Nawroth PP. (1997). *Thromb. Haemost.*, **77**, 772–782.

Brach MA, Hass R, Sherman ML, Gunji H, Weichselbaum R and Kufe D. (1991). *J. Clin. Invest.*, **88,** 691–695.
Chen H, Zhang ZS, Zhang YL and Zhou DY. (1999). *Anticancer Res.*, **19,** 3675–3680.
Chen HW and Huang HC. (1998). *Br. J. Pharmacol.*, **124,** 1029–1040.
Chendil D, Das A, Dey S, Mohiuddin M and Ahmed MM. (2002). *Cancer Biol. Ther.*, **1,** 152–160.
Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC and Hsieh CY. (2001). *Anticancer Res.*, **21,** 2895–2900.
Dixon EP, Stephenson DT, Clemens JA and Little SP. (1997). *Brain Res.*, **776,** 222–229.
Dong Z, Lavrovsky V and Colburn NH. (1995). *Carcinogenesis*, **16,** 749–756.
Dorai T, Cao YC, Dorai B, Buttyan R and Katz AE. (2001). *Prostate*, **47,** 293–303.
Earnshaw WC, Martins LM and Kaufmann SH. (1999). *Annu. Rev. Biochem.*, **68,** 383–424.
Greenlee RT, Murray T, Bolden S and Wingo PA. (2000). *CA Cancer J. Clin.*, **50,** 7–33.
Hallahan DE, Spriggs DR, Beckett MA, Kufe DW and Weichselbaum RR. (1989). *Proc. Natl. Acad. Sci. USA*, **86,** 10104–10107.
Hockenbery D, Nunez G, Milliman C, Schreiber RD and Korsmeyer SJ. (1990). *Nature*, **348,** 334–336.
Huang MT, Lou YR, Xie JG, Ma W, Lu YP, Yen P, Zhu BT, Newmark H and Ho CT. (1998). *Carcinogenesis*, **19,** 1697–1700.
Huang TS, Lee SC and Lin JK. (1991). *Proc. Natl. Acad. Sci. USA*, **88,** 5292–5296.
Inayat MS, Chendil D, Mohiuddin M, Elford HL, Gallicchio VS and Ahmed MM. (2002). *Cancer Biol. Ther.*, **1,** 539–545.
Janicke RU, Sprengart ML, Wati MR and Porter AG. (1998). *J. Biol. Chem.*, **273,** 9357–9360.
Jee SH, Shen SC, Tseng CR, Chiu HC and Kuo ML. (1998). *J. Invest. Dermatol.*, **111,** 656–661.
Jiang MC, Yang-Yen HF, Yen JJ and Lin JK. (1996). *Nutr. Cancer*, **26,** 111–120.
Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA and Sartor RB. (1999). *J. Immunol.*, **163,** 3474–3483.
Juo P, Kuo CJ, Yuan J and Blenis J. (1998). *Curr. Biol.*, **8,** 1001–1008.
Khar A, Ali AM, Pardhasaradhi BV, Begum Z and Anjum R. (1999). *FEBS Lett.*, **445,** 165–168.
Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P and Flavell RA. (1998). *Cell*, **94,** 325–337.
Kuo ML, Huang TS and Lin JK. (1996). *Biochim. Biophys. Acta*, **1317,** 95–100.
Mehta K, Pantazis P, McQueen T and Aggarwal BB. (1997). *Anticancer Drugs*, **8,** 470–481.
Mohandas KM and Desai DC. (1999). *Indian J. Gastroenterol.*, **18,** 118–121.
Mow BM, Blajeski AL, Chandra J and Kaufmann SH. (2001). *Curr. Opin. Oncol.*, **13,** 453–462.
Mukhopadhyay A, Bueso-Ramos C, Chatterjee D, Pantazis P and Aggarwal BB. (2001). *Oncogene*, **20,** 7597–7609.
Ohta T, Kinoshita T, Naito M, Nozaki T, Masutani M, Tsuruo T and Miyajima A. (1997). *J. Biol. Chem.*, **272,** 23111–23116.
Piwocka K, Zablocki K, Wieckowski MR, Skierski J, Feiga I, Szopa J, Drela N, Wojtczak L and Sikora E. (1999). *Exp. Cell Res.*, **249,** 299–307.
Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S and Howells L. (1999). *Oncogene*, **18,** 6013–6020.
Rao CV, Desai D, Rivenson A, Simi B, Amin S and Reddy BS. (1995). *Cancer Res.*, **55,** 2310–2315.
Reed JC. (1997). *Cell*, **91,** 559–562.
Sentman CL, Shutter JR, Hockenbery D, Kanagawa O and Korsmeyer SJ. (1991). *Cell*, **67,** 879–888.
Sharma OP. (1976). *Biochem. Pharmacol.*, **25,** 1811–1812.
Shaulian E and Karin M. (2002). *Nat. Cell Biol.*, **4,** E131–E136.
Sikora E, Bielak-Zmijewska A, Piwocka K, Skierski J and Radziszewska E. (1997). *Biochem. Pharmacol.*, **54,** 899–907.
Singh AK, Sidhu GS, Deepa T and Maheshwari RK. (1996). *Cancer Lett.*, **107,** 109–115.
Singh S and Aggarwal BB. (1995). *J. Biol. Chem.*, **270,** 24995–25000.
Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T and Tohyama M. (1999). *J. Biol. Chem.*, **274,** 8531–8538.
Van Antwerp DJ, Martin SJ, Kafri T, Green DR and Verma IM. (1996). *Science*, **274,** 787–789.
Wang CY, Guttridge DC, Mayo MW and Baldwin Jr AS. (1999). *Mol. Cell. Biol.*, **19,** 5923–5929.
Wang CY, Mayo MW and Baldwin Jr AS. (1996). *Science*, **274,** 784–787.
Wilkinson S, Gomella LG, Smith JA, Brawer MK, Dawson NA, Wajsman Z, Dai L and Chodak GW. (2002). *J. Urol.*, **168,** 2505–2509.